

Mycological Studies in Methods of Improving the
Antibiotic Yield of Micro-organisms.

being a thesis presented by
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GENERAL INTRODUCTION.

I. Events leading to the discovery of antibiotics.

The first clear scientific description of microbial antagonism was given by Pasteur and Joubert (1877), who observed that ^{if} the anthrax bacterium was cultured in a medium along with "one of the common bacteria", the former made very poor growth and soon died. They went on to describe how the same phenomenon occurred when a suspension of the two organisms was introduced into an animal susceptible to anthrax. Pasteur and Joubert were probably observing the effects of a chemical substance produced by one of the common bacteria in vitro, though this might not have been the cause of the results in the animal.

The term "antibiosis" was first used by Vuillemin (1889) to describe the condition, so common in nature, of one animal preying upon another in order to provide its own food. Ten years later Marshall Ward (1899) applied the term "antibiosis" to microbial antagonism but his lead was not followed and the word seldom found use for many years. About this time a paper was published by Bouchard (1889), which initiated the first phase of work on antibiotics, although the word was not used at this time. Bouchard showed that rabbits infected with anthrax were protected to a considerable degree when they were inoculated with Pseudomonas pyocyanea. Within a few years the products of this organism were being tried in man; ~~and~~ a surge of

research was initiated that continued for twenty years and more. This provided the first example of the treatment of human disease by injection of microbial products from an organism other than that causing the disease. The substance came to be called pyocyanase and was used with varying success in many pathological conditions, until it gradually fell into disuse in the late 1920's. Florey (1949) comments, "These older workers had nearly all the ideas which are too readily supposed to be of quite recent origin - their misfortune was that they happened to be working with a rather toxic antibiotic preparation".

The modern era of antibiotics and their great benefit to medicine may be considered to date from the memorable paper by Fleming (1929). In this he published his observations on the behaviour of a Penicillium contaminant (later identified as P. notatum) growing in a Petri dish in the neighbourhood of colonies of staphylococci. He observed that the colonies situated near the contaminating mould were undergoing lysis and concluded from this that the fungus must be producing a substance inimical to the bacteria. This substance he called penicillin. Several workers later attempted to isolate the active material (Clutterbuck, Lovell and Raistrick 1932) (Reid 1935) but they concluded that it was too unstable to justify further attempts. During the last war further research was undertaken at Oxford, which resulted in the isolation of a stable substance which could be used in clinical trials. The success

of these early trials indicated the need for large scale production of penicillin for the treatment of war injuries.

While the Oxford workers were preparing the material for these trials they used cultures of Fleming's original contaminant and with the relatively primitive equipment and techniques available, the strain yielded them a broth containing between 1 and 2 international units/ml. of penicillin, much of which was lost by the methods of extraction in use at the time (Abraham 1941). Less than ten years later, when the manufacture of penicillin had become a major industrial undertaking of many firms both in Britain and America, broths were being produced regularly containing a thousand times as much penicillin per unit volume as those obtained at Oxford and the extraction loss had been very considerably reduced.

This enormous increase in efficiency was brought about by improving the strain of the fungus used in fermentation, by development of the medium ingredients available to the fungus and by improvements in the technique of fermentation, such as the design of vessels, agitation and aeration rates. It would be difficult to say with certainty which of these three lines of research has contributed most to the increase in industrial production but it is safe to say that strain improvement has played as great a part as any of the others.

A very similar story can be told of the discovery

and development of the antibiotic streptomycin. In 1939 Waksman and his collaborators working at Rutgers University started an ambitious programme embracing the examination of many thousands of actinomycetes, fungi and bacteria in order to test the antibacterial substances produced by them. In 1944 Schatz, Bugie and Waksman published the first paper on streptomycin. From then on, the development of this substance followed closely the general pattern of the work on penicillin. More rapid progress was made in the study of streptomycin through the experience gained with penicillin and also because there already existed large-scale fermentation plant which had been built up for the manufacture of penicillin. The introduction of streptomycin was rather more difficult. Being itself somewhat toxic, it is more difficult to purify than penicillin because it is often contaminated with toxic impurities that must be removed before the antibiotic can be used clinically.

The culture of Streptomyces griseus designated 18-16, one of two original cultures isolated by Waksman, became the progenitor of almost all the cultures used in large-scale streptomycin manufacture. This culture yielded between 100 and 200 $\mu\text{g}/\text{ml}$. of the antibiotic under optimum conditions (Waksman 1949). At the present time in industry, yields of more than ten times this quantity are regularly obtained using strains derived from Waksman's original isolate. This state of affairs has largely been brought about by intensive programmes of strain selection and mutation for higher yielding

isolates. Research into medium development and extraction methods have also played a notable part.

This pattern of greatly increased yields from antibiotic-producing organisms, due to mutation and strain selection, is common to almost all antibiotics which have been found useful enough to justify production on a large scale. Dulaney (1954), who has played such a large part in the production of high-yielding streptomycin strains, considers that this method of strain selection and mutation is the most efficient way of increasing yields of "microbially derived pharmaceutical products". It is to this aspect of research and to the development of more efficient methods of carrying it out that this thesis is dedicated.

II. The development of the technique of mutagenesis.

Mutation may be described as the inception of a heritable variation. It is the modus operandi of the process of evolution and the mechanism whereby new material is introduced so as to be worked on by the forces of natural selection. It occurs in nature and indeed until comparatively recently nature was the only source of mutations available to the geneticist. Less than thirty years ago Muller (1927,1928) discovered that he could increase the natural mutation rate observable in Drosophila by the use of X-rays. This discovery opened up the possibility of shaping the heredity of plants and animals.

Mutations do not occur very frequently in nature. It has been calculated that many genes have a mutation

rate in the order of 1 in 10^6 individuals (Huxley 1942) (Stadler 1932). Spontaneous mutations have for a long time been observed to be random, that is to say the *direction of the changes produced by them appear to be unrelated to the* direction of evolutionary change or to the functional needs of the organism. The directive force is imposed upon this random series of changes by the sifting and guiding force of natural selection.

Following Muller's discovery geneticists turned their attention towards other means of inducing mutations and within a few years Altenburg (1930)(1933) had shown that ultraviolet irradiation possessed mutagenic properties. In 1942 Auerbach and Robson reported to the Ministry of Supply on the action of mustard gas in producing mutations in Drosophila. This report, for security reasons, was not made public until after the war (1946, 1947), by which time these authors had successfully tried other related chemical compounds. During the war years Oehlkers (1943) succeeded by chemical means in inducing mutations in Oenothera using a variety of substances. A further independent discovery was published in 1946 by Hadorn and Niggli, who found that phenol could produce high mutation frequencies in Drosophila. Since these early days a vast number of other chemicals have been added to the list of mutagenic agents.

All these discoveries, however, showed that the types of mutations they produced were random and this fact, broadly speaking, holds to this day. Furthermore, there has been found to be a striking similarity between

the mutations caused by radiations and by chemical means. A very complete summary of the position regarding chemical mutagens as it was at that time is given by Auerbach (1949, 1951).

III. Mutation in the service of penicillin production.

As mentioned above, the original Fleming strain of Penicillium notatum yielded very small amounts of penicillin and once the large-scale production of the antibiotic was undertaken attempts were made to find other strains which would be capable of giving more. Up to 1941 a theory was held that this particular culture was unique in its ability to produce the antibiotic. In 1942 a single spore isolate, derived from the Fleming strain, was discovered at the Squibb Institute in America, which gave significantly more penicillin than the parent strain and shortly afterwards a more stable isolate of this Squibb strain, which was designated NRRL 1249 B21, was found. This, together with another P. notatum isolate NRRL 832, both obtained by natural selection were used in the early production of penicillin (Perlman 1950).

At the same time investigations were carried out both in Britain and the U.S.A. in which cultures of P. notatum-chrysogenum and other groups were examined for penicillin production and the majority were found to possess some antibiotic activity (Moyer and Coghill 1946), (Waksman and Reilly 1944). In 1945 Raper working at the N.R.R. Laboratories isolated a very promising strain (NRRL 1951) from a rotting cantaloupe, which as

a wild strain produced nearly as much penicillin as the selected strains then in commercial use. After a period of selection on this strain, a variant designated NRRL 1951 B 25. was isolated which produced more penicillin than those used in industry. (Raper 1945, 1946).

This strain of P. Chrysogenum has become the parent of almost all the penicillin producing strains in use today. So far, the improvements in yield that had been achieved had been brought about by selection from a very large number of species and variants of these species. Continued work along these lines failed to produce any worth while isolates and this may be considered to mark the end of the first phase of strain improvement. During this period considerable gains in yield had been made, although substantial contributions towards the increase in penicillin titres came through the introduction of submerged fermentation in tanks. This change was brought in by the industry between 1943 and 1946.

The position at this time (1946) is summarised in Table I. (taken from Perlman 1950).

TABLE I.

STRAIN	FERMENTATION	YIELD in units/ml	REFERENCE
Fleming's original	Surface	1-2	Abraham 1941
Squibb's NRRL ¹²⁴⁹ B21	Surface precursor	194	Moyer & Coghill 1947
NRRL 832	Submerged (lab. scale.)	141	" " "
NRRL 1951 B 25	Submerged (lab. scale.)	250	Raper 1946

During the next three years two major advances were achieved as a result of mutation combined with selection. Starting with the strain NRRL 1951 B 25. an X-ray mutant was obtained, which was designated X.1612 (Demerec 1948). A further advance was made by workers at the University of Wisconsin, who undertook a programme of mutation involving treating the spores of X.1612 with ultra-violet light. From this process a culture (Wis. Q.176) was isolated, which produced considerably more penicillin than the parent; in fact this was the first culture, which under favourable conditions had yielded in excess of 1000 units/ml. (Foster 1946). The normal average yield of this strain under production conditions was between 600 and 800 units/ml.

The above summary has shown how the mutation and selection of higher-yielding cultures has played its part in increasing the industrial output of penicillin. Since the isolation of Wis. Q 176 many thousands of strains, derived from treating this culture with different mutagenic agents, have been tested, and as a result the yield of penicillin per fermentation has been greatly increased. Many of these strains have been isolated in the research departments of industrial companies and in many cases the only publication available to the public showing the advances made is the patent on the strain. In some cases even this is not available, the company concerned preferring to use their own isolate as a secret process. In this instance the capabilities of the strain in question remain unknown outside the

members of the company. For this reason it is difficult to assess the potentialities of the strains in use in industry throughout the world at this time. It is probably safe to say, however, that the yield obtained from strains in use today approaches three times that of Wis.Q176, namely between 1800 and 2400 units/ml.

This tremendous advance in the productive capacity of the strains in use for pencillin production has been brought about by two mutagenic agents (X-rays and ultraviolet irradiation) coupled with selective techniques which were designed to test the many isolates thus obtained. The whole process of mutation and selection is essentially laborious. This is due partly to the relatively low mutation rates obtainable by these processes, particularly ultraviolet.

It was decided to carry out a programme of research with the object of firstly, developing techniques which would cut down the labour involved in testing very large numbers of strains and if possible to increase the likelihood of obtaining satisfactory mutants and secondly, to endeavour to isolate by these techniques, a higher-yielding strain. This thesis is an account of these investigations.

PART I.

The Use of Sulphur-35 in the Mutation of Penicillium chrysogenum.

Introduction.

Any mutation programme, which has as its object the

increasing of the potential yield of an antibiotic by an organism, is subject to two major limiting factors.

Firstly, there is the inevitable rarity with which the desired mutation occurs and secondly, following on from this, the time and labour taken up by having to test a very large number of isolates so that a satisfactory mutant may have a reasonable chance of being among those tested. Each strain or isolate which is tested must firstly be made to carry out a fermentation in order to produce the antibiotic and then the resulting broth must be assayed to estimate the quantity produced. Unfortunately no correlation has been found between morphological characteristics of Penicillium strains and their potential yield of penicillin. Thus any improvement in the technique of mutation or selection that would cut down the amount of testing involved would materially aid the execution of such a programme.

The normal method of conducting small-scale fermentation experiments with antibiotic-producing organisms has been to ferment them in 250 ml conical flasks, containing 40 - 60 ml. of medium, under sterile conditions. So that the medium may get sufficient aeration the flasks are placed on a mechanical shaker with either a reciprocal or a rotary motion. In the case of Penicillium chrysogenum the fermentation has to be carried out for from 5 to 7 days to achieve the peak titre of penicillin. Thus, a mutation programme involving the testing of thousands of putative mutants necessitates occupying a great deal of shaker-space over

a long period. It was decided that an essential need for any mutation programme involving the selection of higher-yielding strains of P. chrysogenum was to simplify the method of testing the isolates, if possible both with regard to the fermentation and the assay. In addition, if by selecting the mutagenic agent a higher mutation rate could be achieved this would be a potential saving as it could well increase the probability of finding satisfactory mutants.

The development of a surface screening method for testing penicillin mutants.

In the early days of penicillin production the mould was grown on the surface of the medium and the penicillin was harvested by withdrawing the liquid medium and extracting the antibiotic (Abraham et al. (1941)). A simple experiment showed that penicillin-producing organisms would produce the antibiotic when grown on a suitable solid medium (solidified with agar) and that the penicillin diffused into the solid medium as it did into the liquid. Following this, the possibility of screening mutants by a small surface fermentation was studied. If the performance of strains in surface culture could be shown to be correlated with their performance in submerged fermentation it might be possible to carry out a primary screening programme involving a very large number of mutants without the use of elaborate equipment.

Two experiments were conducted to test the feasibility of surface screening as a method. In the first of

these the object was to find out if the medium on which the strains were fermented would affect the correlation between the performance of strains in surface and deep fermentation or whether correlation was independent of the medium used. The second experiment was designed to bring out the degree of correlation (if any) between strains and to indicate to some extent the accuracy of the method.

(a) The effect of the medium on the surface culture performance of various known strains.

Two strains of Penicillium chrysogenum whose performance in submerged fermentation was known to differ considerably were chosen for this experiment. The first was P. chrysogenum X.1612 (see introduction). This strain, an X-ray mutant of NRRL 1951 B 25., was known to have a maximum penicillin yield in deep culture of approximately 200-300 units/ml. The second strain was a subculture of the strain isolated by Foster (1949). This strain, an ultraviolet mutant of the strain known as Q.176, was called by Foster, P. chrysogenum var. brevisterigma and is capable under favourable conditions of yielding some 1500 units/ml. in submerged fermentation. The subculture of this isolate, which has been used extensively in this mutation programme is hereafter called Strain A.

The penicillin production in surface culture of these two strains was tested on the following 16 media (for detail of ingredients and make-up see Appendix A.)

1. Lactobacillus lactis medium.
(tomato juice, dextrose, peptone).
2. Mannitol agar.
(mannitol, Lab-lemco, peptone).
3. Glucose nutrient agar.
(dextrose, beef extract, peptone).
4. Yeast extract agar.
(yeast extract, peptone).
5. Glycerol agar.
(glycerol, Lab-lemco, peptone).
6. E. coli maintenance agar.
(casein digest, glycerol vit. B₁₂)
7. B. subtilis maintenance agar.
(enzymatic casein digest, peptone 'Marmite').
8. Nutrient agar.
(Lab-lemco, peptone).
9. Bennett's agar.
(Bengers casein, beef extract, yeast extract).
10. Meat-potato extract agar.
(pot. ext., beef ext. tryptone, starch).
11. Malt agar.
(Malt extract).
12. Distiller's solubles-soya agar 1
(dist sol., soya-flour, dextrose).
13. Distiller's solubles-soya agar 2.
(same in different proportions).
14. Minimal medium agar.
(simple inorganic salts).
15. Corn steep liquor agar - acid.
(C.S.L., lactose).
16. Corn steep liquor agar - neutral.
(C.S.L., lactose).

These media were chosen, because, with the exception of the last two they had no association with good penicillin production. By this means it was hoped to discover whether the superiority of Strain A. over X.1612 would be manifest in a wide range of media or

whether it was a special phenomenon confined to the media on which the strains had been selected.

Four Petri dishes containing 15 ml. of each medium were prepared (64 in all). Two dishes of each medium were mass-inoculated with a spore suspension of X.1612 and two with that of Strain A. A heavy spore suspension of each strain was prepared by washing the spores off a malt agar slope with physiological saline. One ml. of the required spore suspension was used to inoculate each dish, a sterile glass spreader was used to distribute the suspension evenly over the whole surface of the agar. After inoculation the dishes were incubated for 6 days at 24°C, by which time many of the media were supporting a thick sporing felt of mycelium.

The penicillin produced by these two strains on the various media was assayed by punching a cylinder of agar and mycelium out of the centre of each Petri dish with a sterile No. 4 cork borer (internal diameter 7.0 mm). These cylinders were placed, mycelium side up, on the surface of a B. subtilis assay plate of the large plate type described by Lees and Tootill (1955). As this was in the nature of a pilot experiment, no statistical design was employed in arranging the cylinders on the assay plate, which was in fact plated out in the order shown in ~~Table I~~. ^{list on page 14.} The assay plate was incubated overnight at 28°C and the resulting zones of inhibition around the agar cylinders were measured with vernier calipers. The results are given in Table II.

These results showed that on only half the various

TABLE II

Giving the performance of X. 1612 and Strain A. in surface culture on 16 different media.

Zone sizes measured in millimetres.

1. <u>Lactobacillus</u> stab medium. Strain A. X. 1612 24.0 23.0 29.8 29.5	2. Mannitol agar. Strain A. X. 1612 29.3 28.5 21.8 23.9
3. Glucose nutrient agar. Strain A X. 1612 21.9 22.0 20.0 20.2	4. Yeast extract agar. Strain A. X. 1612 24.7 24.5 20.6 20.9
5. Glycerol agar. Strain A. X. 1612 30.3 31.1 31.4 31.0	6. <u>E. coli</u> maintainance agar. Strain A. X. 1612 26.5 26.6 22.0 22.4
7. <u>B. subtilis</u> maintainance agar. Strain A. X. 1612 Trace, Trace. 15.0 15.2	8. Nutrient agar. Strain A. X. 1612 16.5 16.3 17.2 17.5
9. Bennett's agar. Strain A. X. 1612 25.8 25.6 20.1 19.6	10. Meat-potato extract agar. Strain A. X. 1612 Trace Trace 19.4 Trace
11. Malt agar. Strain A. X. 1612 12.2 12.2 12.9 13.1	12. Minimal agar. Strain A. X. 1612 19.6 19.5 Trace Trace
13. Distillers-solubles soya(1) Strain A. X. 1612 25.2 25.5 24.7 24.3	14. Distillers-solubles soya(2) Strain A X. 1612 24.4 24.4 25.4 25.5
15. C.S.L. agar pH 4.0 Strain A. X. 1612 39.5 39.3 34.9 34.8	16. C.S.L. agar pH 7.0 Strain A. X. 1612 40.6 40.8 34.0 33.4

media used was a potential difference between the strains demonstrated; the two C.S.L. media, as expected, were among them. On three media (1, 7, 8) the order of the strains was reversed, while on four others (5, 11, 13, 14) the strains were shown as potentially equal in penicillin-producing capacity. This experiment brought out clearly the importance of the medium for the selection of mutant strains. The wrong choice of medium could lead to the selection of improved mutants which were in fact capable of higher yields only on the type of medium used in the selection. In order to verify that the reversal of the true potential of the two strains on media 1, 7 and 8 was not accidental, the experiment was repeated with these three media and the acid C.S.L. medium and the same result was obtained.

It is interesting to speculate on the cause of this reversal of penicillin-producing capacity on these three media but it must be remembered that the strain X.1612 is genotypically closer to the wild type P. chrysogenum than is Strain A. X.1612 was selected after one X-ray treatment of a natural variant of the wild type strain isolated from a cantaloupe. Strain A. on the other hand was selected after mutagenic treatment of Q.176 which was itself removed from X.1612 by at least one mutation. It seems likely, therefore, that X.1612 would be more tolerant of environmental changes and its capacity to produce penicillin would be less influenced by changes in the medium than Strain A.

It is clear from the above experiment that any mutant selection programme must be carried out on the medium which it is intended to use in production if spurious selections are to be avoided.

(b) The relationship between production of penicillin by various strains of *P. chrysogenum* in deep fermentation and in surface culture.

An experiment was designed to see if any correlation exists between the known penicillin production of strains in deep fermentation and their performance in surface culture on a solid medium. For this purpose a number of natural variants of Strain A. were obtained from the culture collection belonging to these laboratories. These had been tested in submerged fermentation and their potential titres had been evaluated. Three nitrogen-mustard mutants of Strain A., which had been tested in submerged fermentation and whose performance was known, were also obtained from the same source. These strains together with X.1612 and Q.176 (eight in all) were tested on C.S.L. medium (pH 4.0) solidified with 3% agar.

Four Petri dishes containing 15 ml of C.S.L.-agar medium were inoculated with each strain (32 dishes in all) using dense spore suspensions which were spread over the whole surface of the agar. They were incubated at 26°C for 6 days. Two agar plugs were punched from the centre of each dish and placed on a large assay plate seeded with *B. subtilis*. The design used in plating out the plugs on the assay plate was an 8 x 8

quasi-latin square arranged so that the error between plugs from replicate dishes and the error between plugs from the same dish could be compared.

The results of this experiment together with the known deep fermentation potential of each strain is given in Table III.

The order of strains, determined by their penicillin producing capacity, which was obtained by this surface-screening method showed, with one exception N100, a close correlation to that derived after several accurate tests in shake flasks. The discrepancy caused by the performance of this one strain N100 was, however, disturbing and as a check it was requested that further shake flask tests be carried out. These were done and, harvested after 6 days, titres of 840, 880, and 900 units/ml were obtained on three flasks. This showed that this strain was capable of considerably higher titres than the original estimate and that its position in the order, as found by the surface test was much closer than was at first thought. The zone error of 0.352 mm. was much larger than had been expected but with refinements in technique it was thought that this could be reduced. It was also evident that the error between zones derived from plugs taken from different dishes of the same strain was much greater than that between plugs taken from the same dish.

After considering the experiments so far carried out, it was decided that a primary screening method based on a surface fermentation was feasible and that

TABLE III

The comparison between the performance of certain known strains in deep fermentation and their penicillin production in surface culture.

Strain	Deep fermentation titre.	Corrected zone value, surface culture.	Order shown by surface test.
V.24 [@]	1000 units/ml.	36.5 mm.	3
V.25 [@]	1000 "	35.8 mm.	5
N.82 ^{!!}	500 "	35.48 mm.	6
D.466 [@]	1400 "	36.7 mm.	2
N.100 ^{!!}	150 "	35.9 mm.	4
X.1612	200 "	32.9 mm.	8
N.9 ^{!!}	1500 "	36.74 mm.	1
Q.176	600 "	35.35 mm.	7

@ = Variants of Strain A.

!! = Nitrogen-mustard mutants of Strain A.

Calculated error per zone 0.352 mm.

provided a satisfactory technique could be evolved for handling large numbers of strains, very considerable time would be saved by adopting this method.

Factors affecting the Microbiological Assay of Penicillin using Agar Plugs.

Of the list of factors given by Lees and Tootill (1955a), which influence the zone size in a penicillin assay using large plates, the following were deemed worthy of special investigation, if the agar plug assay was to be used.

- (a) The density of seeding of the assay organism.
- (b) The depth of seeded agar in the assay plate.
- (c) The length of the incubation cycle.

In addition to these, the following factors, not applicable in the normal penicillin cup assay, would have to be studied.

- (1) The effect of variation in plug thickness and diameter,
- (2) The effect of variation in the density of the plug
(e.g. %age agar in the medium).

It was evident from the experiments already conducted on different strains, that using the normal volume of assay medium (400 ml for a 15 inch square assay plate) the zone sizes obtained on a sixth day harvest using plugs would be in excess of 35 mm. Zones of this size would be too large as they would cut down the number of assays that could be accommodated on a plate. As mentioned by Lees and Tootill (1955a) the size of the zone is

inversely proportional to the depth of agar in the assay plate and to the seeding rate of the organism used (B. subtilis). The optimum seeding rate for the organism is usually determined from batch to batch. By doubling the volume of medium in the assay plate and increasing the rate of seeding five times it was found that the average zone size given by Strain A was reduced to about 30 mm, which was a satisfactory size. On investigation, the normal incubation period of 18 hours at 28°C was found to be convenient.

It remained to investigate the effect ~~of~~^{on} zone size of the depth of the punched agar plug. An experiment was carried out in which 15, 25 and 35 ml of 2½% agar containing two levels of penicillin 200 and 50 units/ml was poured into 4 inch (10 cm) Petri dishes. This gave plug thicknesses of 2.6, 4.7 and 6.0 mm respectively. The concentration of penicillin in the plugs was the same for all thicknesses. The plugs were placed on an assay plate containing 350 mls of medium and five times the normal inoculum. The position of the plugs on the assay plate was randomised but no statistical design was used. Four plugs of each type were used giving 24 plugs in all. The results are given in Table IV. Plate incubation was 18 hours at 28°C.

These results show that there is little or no effect on the zone size caused by increasing the depth of the plug. They also indicate that there may be a correlation between plug thickness and the increase in zone size caused by doubling the concentration of

TABLE IV

The effect of variation in plug thickness on the size of the zone of inhibition produced on a B. subtilis assay plate, when the concentration of penicillin remains constant.

Plug thickness	Penicillin concentration in units/ml.	Mean zone size.	Increase in zone size for doubling of concentration.
2.6 mm.	200 u/ml.	24.42 mm.	1.59 mm.
	50 u/ml.	21.24 mm.	
4.7 mm.	200 u/ml.	24.50 mm.	1.63 mm.
	50 u/ml.	21.23 mm.	
6.0 mm.	200 u/ml.	24.52 mm.	1.66 mm.
	50 u/ml.	21.20 mm.	

penicillin. A similar though rather more accurate experiment was carried out by Mr. K.A. Lees (verbal communication) in the microbiological assay department in which an even wider range of plug thicknesses was tested. This experiment was statistically analysed and confirmed the conclusions reached in the experiment mentioned above.

The technique used for the primary screening of mutants by surface culture.

The mutants to be tested were, after isolation, grown up on C.S.L.-agar slopes in universal containers. The slopes were incubated for 8 - 10 days at 24°C. and then placed in the refrigerator until required.

A large number of small metal cups were obtained from Metal Box Co. Ltd. These were of thin aluminium, 32 mm. in diameter and 23 mm. deep, with a loose-fitting lid, and were used as small Petri dishes for the surface fermentation. 3.5 ml. of C.S.L.-agar was placed in these cups by means of an automatic filler-pump set to deliver this volume of medium. After a trial filling of a batch of 100 cups, 90% of these were found to contain between 3.25 and 3.75 ml. of medium, the remainder being only just outside these limits. In practice the medium was made up and the 4% agar added in powdered form. The medium was placed in a stirred reservoir to keep the powdered agar in suspension and in this form it was pumped into the metal cups. After the lids had been placed on the cups they were autoclaved at 15 lbs./sq.in. for 1 minute only, after first

steaming them for 7 minutes. This short sterilisation cycle was found necessary because at the natural pH of the medium (4.2 - 4.4) the setting properties of the agar were quickly destroyed at high temperatures. After cooling the cups were placed in the refrigerator where it was possible to store the medium without drying out or cracking for a week if necessary.

When these cups were inoculated, approximately 0.1 ml. of sterile distilled water was placed on the surface of the medium in each cup.

A mass of spores was scraped off the required mutant culture with a wire loop and transferred to the appropriate cup and the spores were spread over the entire surface of the agar using the 0.1 ml. of water to facilitate spreading. The cups were inoculated in batches of 98 (two sets of 49) two cups being inoculated from each of 48 mutants and two cups from Strain A. to act as controls. (The choice of statistical design used in the assay is discussed in the following section). It was found that contamination of the cups was very rare even though the lids might be removed for as long as a minute during the addition of water and the inoculum. The cups were placed on trays in two 7 x 7 blocks in the randomised order in which they were to be placed on the assay plate. Incubation was carried out at 24°C for 6 days, after which they were assayed.

The assay was carried out as follows. Two 18 inch square glass assay plates, constructed as described by Lees and Tootill (1955) were levelled by placing them

on levelling screws and 800 mls. of B. subtilis medium seeded with this organism were poured into the plates and allowed to set. When the medium had completely set, plugs punched from the test cups and the control were placed on the agar according to the previously randomised arrangement of the statistical design, which was, as already mentioned, their order in the tray. This was done in order to obviate having to number the cups individually. Thus, each assay plate carried a 7 x 7 block of plugs consisting of 48 mutants under test and one plug of Strain A as control. Any plug could be identified by its position on the plate and the randomisation scheme for that plate.

The assay plates were incubated for 18 hours at 28°C after which the resulting zones of inhibition were measured with vernier calipers.

Statistical Conditions in the Design of the Assay Plate.

Previous preliminary experiments had shown that the assay plates were subject to three sources of error. Firstly, there was an error of drift caused by variation in the thickness of the agar across the plate and also due to differences in the seeding rate of the assay organism within the agar. This source of error, with great care in the levelling of the plates and careful mixing of the organism seeded into the agar, could be reduced considerably but even so it always remained a definite source of error. Secondly, there was an error of apparent drift due to the time interval in plating out the agar plugs on the plate. The third source of

error was the residual random error of the zones themselves.

In the surface fermentation there was an error due to the variation in diffusion of the penicillin through the agar in the cup, which was found to be so small that it could be safely neglected. A further error in the fermentation was due to the difference between replicate fermentations; this was not negligible and is the error on which comparison between strains is based. This error was larger than the residual error per zone but not necessarily greater than the drift and time effects. Consequently, provided drift and time effects were eliminated by a statistical design the differences between zones derived from replicate fermentations would be a good measure of fermentation error.

The usual statistical design used, when there is likely to be a drift in an experimental layout, is the Latin Square. This, however, suffered from the disadvantage - as far as this programme was concerned - that the replication required was too high. An increase in the number of replicates would seriously cut down the throughput which was an important factor in the designing of the technique. As precision was not of primary importance so far as the primary screen was concerned, a design was sought which would demand the minimum replication consistent with allowing for a correction for drift. Thus it was decided that Lattice squares best suited these requirements. The design

used was two of the four 7 x 7 squares given by Cochrane and Cox (1950) Plan 12.4 p.361. The size 7 x 7 was used purely for convenience in handling and two plates were set up for each experiment instead of four because it was realised that this would provide the degree of precision required.

Criteria for Selection of Mutants.

Statistical examination of two trial experiments which were carried out using un-mutated isolates of Strain A showed that by using this technique of primary screening it was reasonably certain that any mutants showing a 25-30% improvement on the parent strain would be shown up and that there was a reasonable possibility of mutants showing a 20% improvement being picked out. It was therefore decided that in conducting the primary screening any strain whose corrected zone size fell outside the 95% limits of Strain A should be considered to be a mutant with regard to titre. It was found in practice that the 95% limits of Strain A were calculated to be 1.5 mm above and below the corrected zone size.

Choice of Mutagenic Agent and Method of Treatment.

At about the time this work was started Hungate and Mannell (1952) published their paper on the use of Sulphur-35 as a mutagenic agent for Neurospora. This method consisted of providing the mould with a ~~large~~ part of the available sulphur in the medium in the form of the radio-active isotope.* This is assimilated by the mould and some of it goes to the formation of chromosome material. The sulphur atoms decay at the

* APPROX: 1%

rate of between 0.5 and 1% per day with the emission of negative β -particles (half life 87.1 days) to Chlorine-35 (Kamer 1948). It follows that any Sulphur-35 atoms which have been metabolised and become part of the gene mechanism will, if that atom is essential to the gene function, on decay cause a profound disturbance to the normal function of that body.

By using Sulphur-35, under certain conditions Hungate and Mannell claimed to have obtained mutation rates in the order of 15% of bio-chemical mutants. This mutation rate compares very favourably with rates achieved by any other mutagenic agent including X-rays and is considerably higher than anything achieved by ultra-violet irradiation. In addition these authors claimed that as a result of treatment with sulphur-35, biochemical mutants were recovered which were "quite different from previously described radiation-induced types". Apart from these advantages, sulphur-35 is a convenient isotope to use because its radiation is weak, being absorbed by a glass container and it is safe to handle.

It was decided that any mutagenic agent that would give such a high mutation rate would probably be of great value in the mutation programme in question. Experiments were therefore conducted with the parent strain, which it was proposed to use (Strain A) in order to see how little sulphur the mould would tolerate in the medium and yet grow and sporulate. As a result of this investigation it was found that if the minimal

medium of Bonner (1946) was made up substituting $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ for $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and anhydrous FeCl_3 for $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ the Penicillium strain in question would ^{not} grow and sporulate as freely as it did on the minimal with sulphur but enough spores were produced to give a workable suspension.

Following this information an experiment was conducted to find out the order of mutation rate, which could be expected using sulphur-35 as the mutagenic agent and Strain A. as the parent strain. One millicurie of sulphur-35 was obtained from A.E.R.E. Harwell, in the form of carrier free H_2SO_4 (volume 0.63 ml.). 1 ml. of sulphur free minimal medium solidified with 1% agar ^{*} was placed in each of several 25 ml. conical flasks plugged with cotton wool. The $\text{H}_2\text{S}^{35}\text{O}_4$ was neutralised with NaOH and the volume made up to 2.0 ml. for greater accuracy in distribution. 0.4 ml. of this solution was transferred to five of the flasks containing sulphur free minimal medium (0.2 millicuries/flask) while 0.4 ml. of distilled water containing the same quantity of Na_2SO_4 was transferred to five control flasks. All the flasks were then autoclaved at 15 lbs/sq. in. for five minutes and allowed to cool and set. A conidial suspension of Strain A. was prepared; a loopful of spores being scraped off a malt agar slope and placed in 3-5 ml. of sterile distilled water. Care was taken that none of the malt agar medium was transferred. Two drops (circa 0.15 ml.) of this ^{spore} ~~slope~~ suspension ^{were} ~~was~~ transferred to the surface of the sulphur-35 and the control media and spread over

** It is realised that agar contains sulphur but it is doubtful if it is available to the mould.*

the surface by agitation. Both sets of flasks were then incubated at 24°C . for 8-10 days. During this time the spores germinated and by the fourth day a mycelial mat had covered the surface of the medium. By the seventh or eighth day the mycelium had sporulated and growth had stopped owing to exhaustion of the medium. The flasks were then transferred to a refrigerator until required.

Flasks were harvested 20 and 54 days from the time of inoculation. After 20 days two each of treated and control flasks were taken and the spores were washed off the medium with 5 ml. of Calsolene in distilled water and a few sterile glass beads. The spore chains were broken up by vigorously sucking and squirting the suspension through a Pasteur-pipette for about 15 minutes. Haemocytometer counts were carried out on the spore suspension from each flask, which resulted in the following counts:

Sulphur-35 treated (1) 2.5×10^6 spores/ml.

(2) 3.0×10^6 spores/ml.

Control (1) 3.5×10^6 spores/ml.

(2) 3.8×10^6 spores/ml.

The spore suspensions were diluted so that when 0.1 ml was placed on C.S.L.-agar in Petri dishes and spread with a glass spreader it would result in about 150 colonies per plate. Ten plates were prepared thus from each suspension. The control plates after incubation for 4 days at 24°C averaged 306 per plate, while those prepared from the treated flasks averaged 14.5

colonies/plate. From this it was deduced that there had been a 'kill' of over 90% in the treated spores.

Four hundred colonies derived from each flask were isolated on to minimal medium in order to find out the number of nutritional mutants which had been formed. The method used was essentially that described by Beadle and Tatum (1945). The final percentage of established nutritional mutants found in the treated flasks was 3.75% and 3.5%; no nutritional mutants were isolated from the control flasks. The same procedure was adopted on the 54 day harvest, after which time the killing rate was found to be 98.5% and the nutritional mutation rate 4.25 and 4.75%. One nutritional mutant was isolated from one control flask.

These results, while falling considerably short of the nutritional mutation rate achieved by Hungate and Mannell for Neurospora (1952) compared very favourably with previous results achieved by ultra-violet irradiation in this laboratory. Using ultra-violet irradiation from a Hanovia model XI filter-jacketed tube giving 90% of its energy (1700 ergs/cm^2) at a wavelength of 2537\AA , and having the sample 30 cm from the tube, the highest nutritional mutation rates achieved were in the region 0.75-0.85%, using the same organism (Strain A). The killing rates when ultra-violet ^{was} ~~were~~ used were between 90-95%. As a result of these experiments it was decided to use Sulphur-35 as the mutagenic agent in this programme.

Results of the Mutation Programme.

Using the technique and method outlined above spores of Strain A were harvested after treatment for 18, 36, 54 and 72 days with Sulphur-35. It was early apparent on testing these isolates that something new had been achieved regarding the number of strains which had mutated in respect of their penicillin titre. Of the first five batches of mutants that were tested it was found that ~~in~~ 31, 30, 32, 20 and 34 isolates out of the 48 tested, an average of about 60%, fell outside the 95% limits of the parent (Strain A). These, by previous definition, were considered mutants. The great majority of these isolates had had their titre reduced by the mutagenic treatment but some 2% were showing an increase above the 95% range, which was a percentage far higher than anything that had been achieved in this laboratory with either ultra-violet or nitrogen-mustard as mutagenic agents.

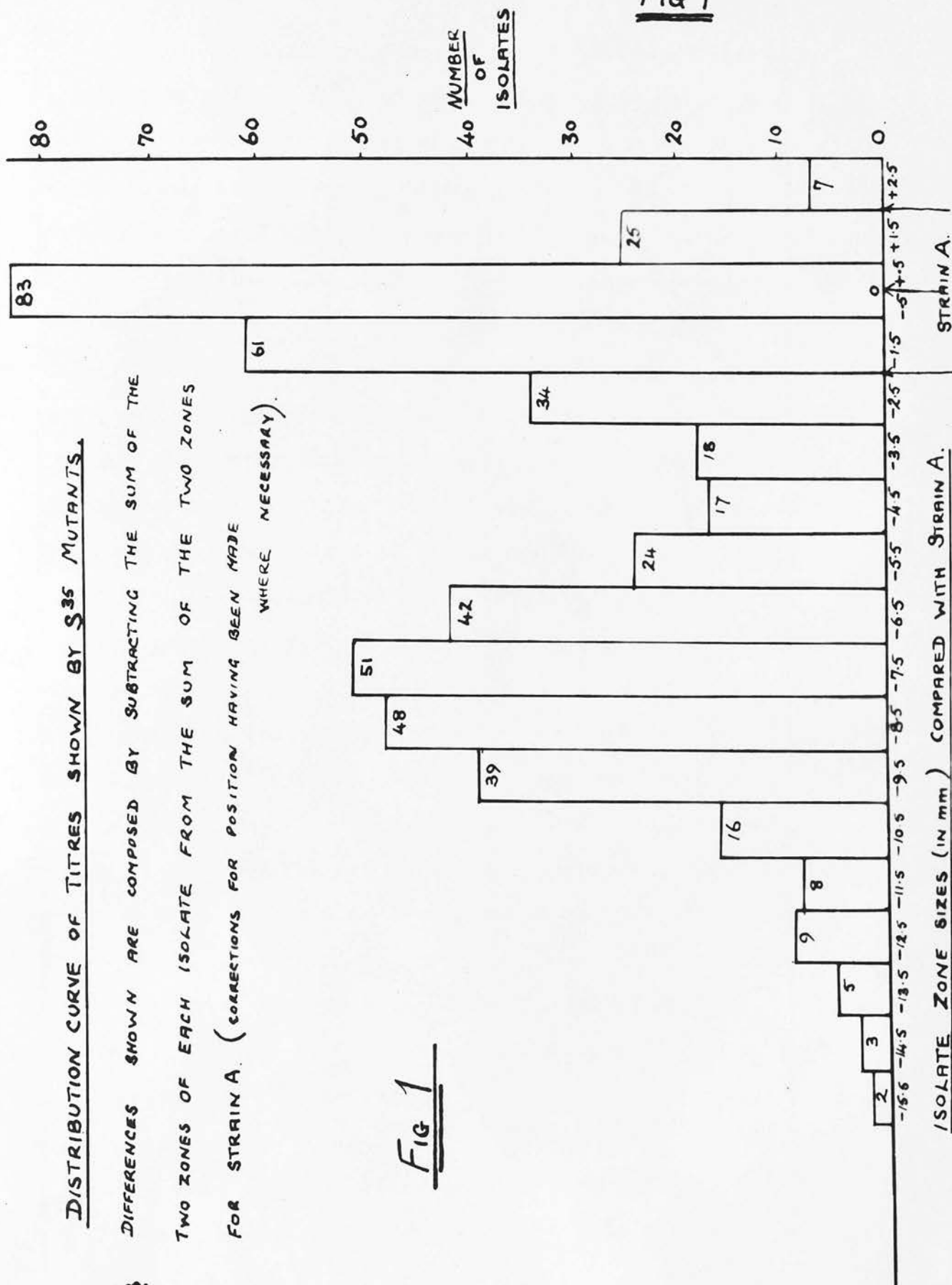
The distribution of the strains obtained by sulphur-35 mutation is shown in Fig. 1. This histogram records the frequency with which strains giving varying performances on the surface screen were encountered. The figure shows, as would be expected, a peak frequency in the neighbourhood of the control, presumably representing strains negligibly affected by the mutagenic agent, and a small frequency significantly in excess of the control, performance which represents genuine favourable mutants together with a number of apparent mutants falsely chosen by the risk level accepted (see p.24). The histogram further shows a number of decreased-titre mutants, a

DISTRIBUTION CURVE OF TITRES SHOWN BY S³⁵ MUTANTS.

N.B

DIFFERENCES SHOWN ARE COMPOSED BY SUBTRACTING THE SUM OF THE TWO ZONES OF EACH ISOLATE FROM THE SUM OF THE TWO ZONES FOR STRAIN A. (CORRECTIONS FOR POSITION HAVING BEEN MADE WHERE NECESSARY).

Fig 1



BASED ON PERFORMANCE OF CONTROL STRAIN.

phenomenon expected in any mutation programme, but these lower titre mutants are not distributed at random. There is an aggregation around -7mm , a titre that corresponds very closely with the performance of Strain X1612, a progenitor of the control strain (see General Introduction). The data thus support~~s~~ the hypothesis that a considerable proportion of the reduced-titre mutants can be regarded as instances of sulphur-35 having, as it were, undone the beneficial effects of earlier mutations. The 492 isolates used to make up this histogram consisted of 230 cultures isolated after 18 day sulphur-35 treatment and the remainder were 36 day sulphur treated strains. The distribution curves of these two treatments, when composed separately, both showed the aggregation around the one value.

After 500 isolates had been screened it was found that eleven had shown up higher than the 95% range of the parent. The five best of these were chosen for trial in shake flasks and 5-litre fermenters. The results of the primary screening on these five isolates are given in Table V.

These five strains were set up in 250 ml conical flasks on a rotary (2 inch throw) shaker in C.S.L.-lactose medium, three replicate flasks being prepared for each strain to be tested and for Strain A used as control. The flasks were inoculated with a vegetative inoculum and were assayed after 5 and 6 days on a B. subtilis plate.

At the same time the 5 strains were fermented in

TABLE V.

Showing the performance in surface culture of the five best mutants as demonstrated by the primary screen.

ISOLATE NO. CORRECTED ZONE SIZE. INCREASE ON 95%
LIMITS OF CONTROL

S.4	29.8 mm.	0.6 mm.
S.67	29.4 mm.	0.8 mm.
S.119	30.7 mm.	1.0 mm.
S.234	30.4 mm.	0.5 mm.
S.483	30.0 mm.	0.5 mm.

N.B. All these isolates were screened in separate batches.

TABLE VI.

Showing the performance of the same five strains as above on the secondary screen.

STRAIN NUMBER	250 ml. flask fermentation		5-litre fermentation.	
	118 hrs.	142 hrs.	118 hrs.	142 hrs.
S.4	1220	1480	1600	1970
	1190	1500		
	1290	1480	1510	1910
S.67	1510	1740	1710	2010
	1550	1660		
	1490	1620	1740	2050
S.119	1250	1490	1650	1810
	1100	1600		
	1190	1460	1660	1900
S.234	1300	1440	1500	1750
	1280	1500		
	1260	1510	1590	1750
S.483	1100	1320	1200	1690
	1200	1300		
	-	-	1430	1770
CONTROL STRAIN A.	1160	1460	1350	1710
	1130	1540		
	1200	1560	1390	1660

All titres given in units/ml.

5-litre fermenters in C.S.L.-lactose medium, two fermenters being prepared for each strain being tested and Strain A. as control. These vessels were fermented for 144 hours, and samples for assay were withdrawn at 118 and 142 hrs. for assay on B. subtilis plates. The results of these two test fermentations are given in Table VI.

The results of these tests showed that the strain S.67 (henceforth called Strain B). was outstanding in both types of fermentation, being 10% better than the control in shake-flasks and 19% better in the large units at the second assay. Of the other four strains tested, two (S234=S483) did not show any improvement on the control. It must be remembered, however, that as the 95% limits of Strain A. were used as the criterion for a mutant, five strains in every hundred of the unmutated parent were liable to be considered as mutants and some of these were likely to fall on the higher side of the 95% range of the parent. It was decided on the evidence of these results to concentrate on testing the mutant Strain B. as being the one most likely to repay development.

It became evident during the subculturing of these five strains that while they were growing up on C.S.L.-agar slopes there was a marked tendency for all the strains to produce sectors. ^(see PLATE I A) This might have been due to the fact that they were mixed isolates, i.e., that they had not in fact been each descended from a single spore; but it was more likely as all the strains showed the

same tendency that a small quantity of unchanged sulphur-35 had been transferred in the subculturing and that this was in fact causing further mutations to take place. To eliminate this instability in the chosen mutant (Strain B.), giant colonies were grown on malt agar from point inoculations of a few spores placed in the centre of a Petri dish. Ten such dishes were prepared and incubated at 24°C. After 21 days the colonies averaged about 40-45 mm. in diameter and each one contained from 10 to 20 well defined sectors of varying growth rates and morphological characteristics. In all, 85 of these sectors were isolated by removing fragments of mycelium from the very edge of the colony and transferring them to C.S.L.-agar slopes. These sector isolates were treated as if they were mutant isolates and tested on the primary screen in the normal way.

As a result of this second screening 44 sector isolates showed up as having lower titres than the control (Strain A.) and by the criterion adopted (95% limits of Strain A.) were classed as mutants. Two sectors, Nos. 55 and 60 were considerably higher than Strain A., the remainder fell within the 95% limits of the parent strain. A further series of giant colonies was prepared from No. 55 and No. 60 and whereas No. 60 now appeared stable (producing only one doubtful sector in 5 plates), No. 55 still showed a tendency to produce sectors and was put aside for further purification if necessary. The isolate No. 60 (henceforth called B/60)

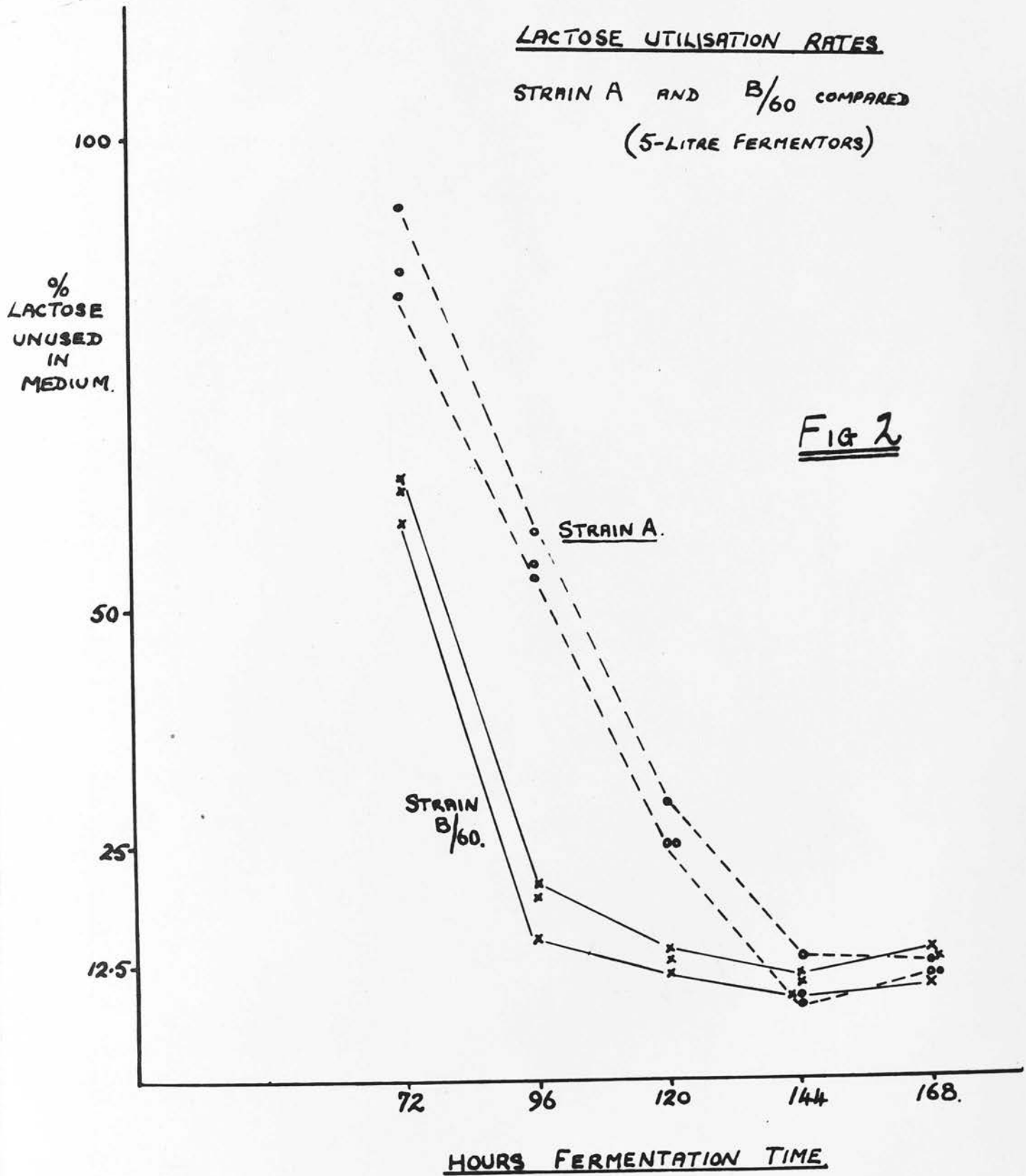
was tested in shake-flasks (four replicates) and in 5-litre fermenters (three replicates) on C.S.L. medium, using Strain A. as control.

The results of the experiment in shake-flasks were somewhat lower than for Strain B. The four replicate flasks of B/60 on average showed a 7% increase on the control. At the 5-litre scale, however, the new strain showed a 28% improvement on the control at the harvest assay (142 hrs.). Furthermore, samples of the fermentation broth taken at 24-hourly intervals were sent to the chemical assay laboratory for determination of the lactose level in the ~~medium~~^{medium}. This analysis showed that the lactose in the medium was being used up more quickly by the mutant than by the parent strain. These results are given graphically in Fig.2 and show that in the mutant fermentation the lactose was exhausted 24 hrs. earlier than in the control fermentation. This was the first intimation, other than the improvement in penicillin titre, of any physiological difference in the metabolism of the two strains.

While these trials were being carried out on Strains B. and B/60, further batches of mutants, harvested after 36, 54, and 72 days treatment were tested on the primary screen. The percentage of mutants obtained after the longer treatments was about the same as that found after shorter treatments, but the percentage of isolates giving higher titres than the parent strain dropped from about 2% after the 18 day treatment to about 0.25% after 72 days treatment. This feature is discussed later.

LACTOSE UTILISATION RATES

STRAIN A AND B/60 COMPARED
(5-LITRE FERMENTORS)



Those mutants which in the primary screen showed themselves better than the parent strain were tested in shake-flasks but none was found to be as good as the original Strain B.

Further Development of Strain B/60.

At this stage samples of broth from B/60 fermentations were sent for chemical assay to confirm that the type of penicillin produced by this strain was in fact penicillin G. When this was confirmed the development of the mutant B/60 was carried further both in shake-flasks and 5-litre fermentors but this work falls outside the scope of this thesis. Briefly, however, an optimal medium was found for B/60 which was different from the optimal medium required by the parent. The mutant, in general, required a rather richer medium than the parent.

Mutation of Strain B/60 using Sulphur-35.

During these trials a further mutation programme was initiated in which B/60 was used as the parent strain and treated with Sulphur-35 using the same procedure as before. In this second programme the mutation rate for penicillin titre averaged rather less than when Strain A was treated (50-60%), but it was felt that this change might have been brought about by differences in the amount of contaminating sulphur present as impurities in the material used. The procedure adopted was unchanged except that a new batch of sulphur-free minimal medium had to be prepared and a new supply of $\text{H}_2\text{S}^{35}\text{O}_4$ was obtained from A.E.R.E. Harwell.

As a result of this treatment between 800 and 900

isolates were tested on the primary screen. These were harvested after 20, 40 and 60 day treatment with sulphur-35. Eleven isolates were selected from these as being better than the parent strain B/60. Seven of these eleven were obtained from the 300 isolates (approx.) harvested after 20 days treatment, the remainder being from the 40 day treatment. None was obtained from the 60 day treatment. Of these isolates, which were tested in shake-flasks, one, designated Strain C, showed an improvement of 11% on the parent (Strain B/60); of the others, three showed an improvement of from 4-8%. The remainder did not show any noticeable improvement. Strain C was tested at the 5-litre scale but the improvement on the parent (B/60) in these fermentations was small.

Subsequent development of this strain (not carried out by the author) revealed that it was substantially similar in penicillin-producing capacity to the parent but it did not require a richer medium to attain this level. The optimal medium for this mutant was almost identical with that required by Strain A and the lactose utilisation rate was identical.

At this point it was decided to discontinue the investigation on treating Penicillium Chrysogenum and to turn to another species. Both these mutants might on further development be suitable as production strains. The work so far carried out has shown that sulphur-35 is a powerful mutagenic agent and induces high mutation rates for titre in certain penicillin producing strains.

As is well known the molecule of penicillin contains a sulphur atom and it is reasonable to suppose that some of the enzymes concerned in the bio-synthesis of penicillin may contain sulphur. The mutagenic action of sulphur-35 may affect some genes concerned in the control of these enzymes. If work was carried out on a mould producing an antibiotic not containing sulphur some information might be gained as to whether there is anything specific in the action of the mutagenic agent or whether the mutations produced were completely at random.

Conclusion.

In assessing the results of this screening programme and the efficiency of the various techniques which had been used, one must consider first the surface screening method. This method was adopted to allow more strains to be tested than would have been possible if the more conventional shake-flask method had been used. When this programme was under way the author noticed a paper by Arima (1950) in which he describes a surface screening method for selecting penicillin producing mutants. One of the conclusions Arima came to was that his method lacked the accuracy needed to select strains with small differences in titre, 'But it is satisfactory for selecting strains showing considerable advances in titre, whether obtained by natural selection or by induced mutation' (translation). Unfortunately he does not specify the order of increase in titre that he considered to be 'considerable'.

The primary screening programme developed for this mutation programme differs in several ways from that used by Arima (1950).

(1) Medium. Arima states that he tried four different media including a corn-steep medium for growing his test strains. His Table V clearly shows that the corn-steep medium gave him his largest zones i.e., the greatest yield of penicillin. In spite of this he says 'As B (Czapek +2% peptone medium) was a good medium it was preferred'. This must be considered to be a weak point in his argument. It is well known that, apart from the very early days of penicillin manufacture, corn-steep liquor has been the medium ingredient chosen in industry. Because of this the strains used in production of the antibiotic have been selected because they were good producers on this medium. It has been clearly demonstrated above and Dulaney (1954) supports this fact that strains which have been selected for superior performance on one type of medium do not necessarily show that improvement and may even be low yielders on another type of medium. The medium chosen by Arima (Czapek +2% peptone) is virtually a synthetic medium and could not be considered in any way similar to the corn-steep medium.

Later in his paper Arima (1950) investigates the correlation between the performance of strains in surface and in submerged fermentations. He states that in an experiment using Q176 and 16 monospore isolates derived from ultra-violet treatment 'The coefficient of

correlation is rather small'. It seems very probable that this is due to his choice of medium for selection. His shake-flask fermentations were carried out in corn-steep medium and it is likely that many of his isolates would not repeat the performance on this medium that they had shown on the surface culture medium. The method adopted by the author, in which the primary screening was carried out on the medium intended for use in subsequent fermentation is considered to be an improvement and the correlation between the performance in the two methods of fermentation in this work has been good.

(2) Method of growing surface cultures. Arima, in his surface culture technique, used the point method of inoculation in the centre of a Petri dish and he punched his agar cylinders from a position outside the edge of the colony. In preliminary experiments, already described, the method of seeding the entire surface of the agar and punching the cylinder from the centre of the cup was found to give more consistent results and was preferred. This, however, is not a fundamental point.

(3) Size of punch. It is felt that the size of punch used by Arima as stated in the paper $55 \pm 1 \text{ mm}$ is a misprint, as a plug of this size would be quite unmanageable. The size used was probably $5.5 \pm 0.1 \text{ mm}$ which is close to the punch used in this programme (No. 4 cork-borer $7.2 \pm 0.1 \text{ mm}$ internal diameter).

In spite of these differences in the two surface screening methods there is a considerable measure of

agreement on the basic principles which apply to the two methods; apart, that is, from the decision as to the choice of medium made by Arima, which must be questionable. The fact that he does not record the selection, by his method, of any strain showing improved titre on corn-steep medium must be largely due to this choice of medium.

The surface screening method as used in this programme has proved successful in that it has been able to distinguish strains which show improvement in the order of 20-30% and it may be safely assumed that had strains been tested which had greater potential improvements on corn-steep medium they would have been picked out by this method. The technique of surface screening has allowed a larger number of strains to be tested in the time than would have been possible in deep fermentation using mechanical shakers. It is true that spurious mutants have been selected but this was expected and allowed for at the outset. The choice of the 95% limits of Strain A which was the criterion decided upon in defining a mutant allowed a possible 5% of unmutated isolates of Strain A to be selected for further testing. In addition the use of surface culture incurred the risk of selecting strains which had mutated in such a way that their yield in surface culture only had been improved. Such strains were found and picked out by this method but they constituted only a small proportion of the mutants and they were certainly not frequent enough to invalidate the method. In practice some

10-15% of the mutants selected for further treatment appeared to be specially adapted to surface culture, thus giving a total wastage of selected strains of about 20%. This was considered to be a reasonable wastage.

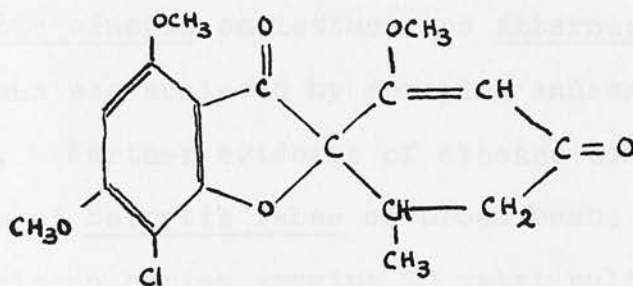
PART II.

Sulphur-35 and Ultra-violet Irradiation in the Mutation of *Penicillium patulum*.

Introduction.

In 1939 Oxford, Raistrick and Simonart published a paper describing the isolation of a chlorine-containing metabolic product from the mould *Penicillium griseofulvum* Dierckx., for which they proposed the name griseofulvin. They studied the chemistry of the substance and described a number of its derivatives and degradation products and postulated a structural formula. No intimation was given, however, of any biological activity. Several years later, Brian, Curtis and Hemming (1946) described the isolation of an antibiotic from a culture of *Penicillium janczewskii* Zaleski, that possessed the property of causing a distorting and curling effect on the hyphae of *Botrytis allii* Munn., and which they called "curling factor". This paper mentioned its fungistatic properties and gave a method of production, extraction and assay. Shortly afterwards, the identity of "curling-factor" with griseofulvin was established by Grove and McGowan (1947) and by Brian, Curtis and Hemming (1949).

The chemistry of griseofulvin has been fully worked out by Grove et al. (1952a,b,c,d,), Mulholland (1952a,b,), MacMillan (1953,1954), and MacMillan et al (1954). As a result of these investigations the structural formula has been established which differs somewhat from that originally put forward by Oxford, Raistrick and Simonart (1939). In brief, griseofulvin is a colourless, crystalline, neutral compound M.P. 220°C. having the following structure (Grove et al. 1951).



MacMillan (1951) was able to identify in culture filtrates of P. janczewskii and P. griseofulvum a substance which he called dechloro-griseofulvin. This compound has the same molecular formula as griseofulvin except that the chlorine atom is replaced by one of hydrogen. Dechlorogriseofulvin also has the property of inducing curling and stunting of B. allii hyphae but it is much less active. Griseofulvin is slightly soluble in water but is soluble in a number of organic solvents including chloroform, n-butyl alcohol, acetone and ether. More recently Ashton and Rhodes (1955) have found that dimethylformamide is also a useful solvent. Griseofulvin is thermostable and it can safely be autoclaved, (Brian, Curtis and Hemming 1946).

The biological activity of this antibiotic has been determined by Brian (1949) who found that it showed

fungistatic activity towards a very wide range of fungi but that it had no effect on yeasts, bacteria or actinomycetes. The activity, according to Brian's theory, is restricted within the fungi to those that possess chitinous cell walls, but this view must be modified as a result of the work of Napier, Turner and Rhodes (in press). A new interest in griseofulvin was aroused by Brian, Wright, Stubbs and Way (1951) when it was shown to become systemic in plants and that good control of Botrytis cinerea on lettuce and Alternaria solani on tomatoes was achieved by spraying and watering respectively. Further evidence of disease control in plants, namely of Botrytis fabae on broad beans and Erysiphe graminis on barley growing in water culture, is mentioned by Brian (1952). Stubbs (1952) using his own technique for evaluating systemic fungicides (Alternaria solani on tomatoes) compared griseofulvin with two nitrosopyrazoles and trichlorophenoxyacetic acid. Of these he found the antibiotic to be markedly the most effective in controlling the pathogen. Up to the present the investigations which have been carried out (summarised by Brian 1954) have tended to confirm the promise shown in the earlier work and it has become evident that griseofulvin might become a useful weapon to the plant pathologist.

One of the chief obstacles in the way of large-scale trials has been that up till recently there have been no reports of griseofulvin being produced other than by the surface-culture method developed by Brian,

Curtis and Hemming (1946). This method does not lend itself to large-scale production and insufficient material has been available for field trials. During the past year a process has been developed in these laboratories for producing griseofulvin in submerged fermentation (British Patent Application No. ~~5012~~¹⁴⁰⁸⁴/55) and the antibiotic has become available in greater quantity. This has allowed field trials to be planned to investigate its uses in the control of plant diseases on a larger scale. With the development of a submerged-culture technique for producing griseofulvin, the need was soon felt for a strain which could produce it in greater yield and a mutation programme was planned with the object of producing such a strain. It was decided that this programme would be a satisfactory one in which to continue the investigation of the mutagenic properties of radioactive sulphur (S^{35}).

At this time Farrell (1953) published a paper in which she described the marked success she achieved in the mutation of P. chrysogenum Wis. Q.176 for higher penicillin-yielding strains. The technique she used was essentially a sequential one. This was carried out by isolating a small number of survivors (20-30 in number) from an ultra-violet irradiated spore suspension and testing for penicillin production in one fermentation using corn-steep medium. The isolate producing the highest titre in this fermentation, irrespective of statistical significance of the increase, was grown up in culture and the spores irradiated and the same procedure was repeated. At the end of six irradiations

and selections Farrell had obtained a strain showing about 100% improvement on the parent strain. It appeared that this technique was worthy of trial in the griseofulvin mutation programme which was being organised. Consequently a simultaneous mutation programme was planned consisting on the one hand of ultra-violet irradiation and selection as described by Farrell (loc. cit.) and on the other hand a sulphur-35 treatment of spores similar to that described in Part I of this thesis.

This arrangement was not intended to be a comparison of the efficiency of the mutagenic agents concerned, because there would be no real basis on which such a comparison could be made. The isolation of one or two desirable mutants using one technique and failure to isolate any by the other could be fortuitous. Again, hardly enough is known of the action of mutagens to be certain that both were being used under optimal conditions. The only comparison which would be possible in this case would be between the mutation rate given by one mutagen as used under one set of conditions and the mutation rate of the second as used under different conditions, but the value of such a comparison would be doubtful. Both methods were used in this programme because they have been shown to be efficient techniques and capable of producing desirable mutants in a relatively short programme. In an extreme case, for example, if one of the mutagenic agents were to produce large numbers of one particular type of mutant, this fact

would be noticed in a simultaneous programme such as this but as both mutagens are known to induce a great variety of mutant types this eventuality would be remote.

The object of this programme was to produce a strain of Penicillium patulum giving a higher yield of griseofulvin than the parent and also to study the action of the mutagenic agents used in the attainment of this object and if possible to form some opinion as to their relative place among those mutagens which have been used successfully to increase the antibiotic yield of micro-organisms.

Studies in the effect of griseofulvin on the hyphae of Botrytis spp.

Before starting this mutation programme, a series of experiments was conducted to find out something of the mechanism by which griseofulvin has its effect on fungal hyphae.

A paper reporting the results of this investigation has been accepted for publication in Annals of Botany. A copy of this paper is submitted with this thesis. The development of a primary screening method for the mutants of Penicillium patulum.

A. Choice of strain. According to Brian, Curtis and Hemming (1949) griseofulvin is produced by at least three different species of Penicillium belonging to three distinct major divisions of the genus viz:-

P. griseofulvum Dierckx Asymetrica-Fasciculata.

P. janczewskii Zal Asymetrica-Divariata.

P. patulum Bain Fasciculata.

Under the more recent system of classification adopted by Raper and Thom (1949) these authors consider that P. griseofulvum Dierckx. belongs to the P. urticae series (Asymetrica-Fasciculata) of which P. patulum Bain. is but a synonym. P. janczewskii Zal. they find indistinguishable from P. nigricans (Bainier) Thom. The strain of P. patulum that was used in these laboratories for the production of griseofulvin in submerged culture (described in British Patent Application No.9012/55) was chosen as the parent strain from which it was hoped by mutation to produce a higher yielding strain. This strain is designated Strain 1. throughout these investigations.

The biological assay described by Brian, Curtis and Hemming (1946) is based on the fact that griseofulvin causes the characteristic curling effect on growing hyphae of Botrytis allii which is discernible at concentrations as low as $0.1 \mu\text{g./ml.}$ The assay consists of a serial dilution of the griseofulvin-containing material in Weindling's medium and the addition of spores of B. allii. The series of dilution tubes are then incubated overnight at 24°C. The germinated spores are examined microscopically and the dilutions, at which the stunting effect and the curling effect cease, are noted. This assay is unsuitable for routine work dealing with large numbers of samples, both because it is very time-consuming and because in Brian's own words "the result is necessarily over-dependent on subjective influences". At the same time as this mutation pro-

gramme was started, a spectro-photometric assay was developed in these laboratories by Ashton and Brown (1956) and it was thought that this method would probably form the basis of a workable assay for the primary screening of mutants. The assay involves a simple differential solvent extraction of the griseofulvin from the fermentation broth and after suitable dilution the antibiotic is measured by means of the ultra-violet spectrophotometer.

B. Choice of a fermentation method and a medium for the primary screen.

The possibility of developing a surface fermentation, similar to that used in Part I for testing P. chrysogenum mutants, was carefully considered but it was found to be impracticable. A few pilot experiments were undertaken in which agar cylinders punched from Petri-dish cultures of Strain I. were placed in various suitable solvents for varying times, and the amount of griseofulvin measured by ultra-violet spectrophotometer. The results obtained were, however, very inconsistent and although it was felt that a suitable assay method could have been developed for a surface-culture method, the idea was abandoned in favour of a small-scale submerged fermentation. In order to make the maximum use of the shaker-space available the fermentations were carried out in 30 ml. glass vials containing 7.5 ml. of medium. The small quantity of medium available precluded any possibility of serial sampling of the broth for assay at different times and one harvest time had to

be chosen.

At the start of this programme the type of medium most suitable for the large-scale production of griseofulvin was still under investigation and therefore it was not possible to screen the mutants on the medium which would later be used for production. The importance of using the same medium throughout has been stressed in Part I. Until such time as the type of medium to be used in production was known the medium which gave the most consistent results under the conditions of the primary screen fermentation was used.

Brian, Curtis and Hemming (1946) found that the two best media for the production of griseofulvin in surface culture (using Penicillium janczewskii) were Czapek-Dox plus 0.1% peptone and a corn-steep liquor medium. They also found that there was no significant difference in the titres obtained from them. These two media and in addition a nitro-chalk medium were tested in the vials which would be used in the primary screen fermentation. The nitro-chalk medium was introduced because, should a synthetic medium of the Czapek-Dox type be found to be the best medium for large-scale fermentation, the cheapest source of the ingredients in bulk would probably be agricultural fertilisers and this test of the nitro-chalk medium was designed to cover this eventuality.

An experiment was undertaken to find out what order of titres could be expected from Strain I in the 30 ml. vial-fermentation and what degree of reproduc-

ability was attainable using a series of these vials all fermenting the same strain. The experiment was also planned to gain information on the probable length of the griseofulvin fermentation in these vessels.

Eighty four vials were prepared and arranged in three groups of twenty eight. To each group of vials was added 7.5 ml. of one of the three media concerned (Czapek-dox plus peptone, nitro-chalk and corn-steep media). A small scratch was made on the side of each vial at the level of the medium to show the amount of evaporation, if any, which had taken place during the fermentation. Prior to this a 10 oz. bottle containing 40 ml. of Czapek-dox medium solidified with 2% agar was prepared and sterilised. The medium was allowed to set with the bottle laid on one of its broader sides, thus forming a large surface of medium. This surface was inoculated with spores of Strain I and incubated at 24°C. for 8-10 days after which the culture had sporulated freely. A spore suspension was prepared by washing the spores off the mycelium with 30 ml. of sterile water and a few glass beads. All the vials were inoculated with 0.5 ml. of this spore suspension and placed on the shaker (2 inch rotary throw, 120 r.p.m.). It was arranged that seven vials of each medium should be harvested after 5, 7, 9, and 12 days and the broths of each vial assayed. It was estimated that by the 12th day the peak titre should have been reached and that the fermentation would be complete.

The assay procedure, which was a simplified version

of that described by Ashton and Brown (in press) was as follows. The contents of each vial ^{were} ~~was~~ made up to the original mark with distilled water and ~~the contents~~ ~~were~~ tipped into a large boiling tube, any mycelium remaining in the vial being scraped into the tube afterwards. To each boiling tube was added 15 ml. of cyclohexane (at this time considered to be the most suitable solvent for differential extraction) and the tube was closed with a rubber bung holding a 14 inch length of glass tubing to act as a simple reflux condenser. The boiling tubes were placed in a water-bath heated to 70°C. and left for an hour. After this they were removed and 10 ml. of the cyclohexane layer was withdrawn by pipette, diluted if necessary, and set aside for reading on the ultra-violet spectrophotometer against a cyclohexane blank. Using 1 cm. silica cells three readings were taken on each sample at wavelengths of 261.5, 288.0, and 322.5 μ . and the titre values calculated. Reading at these wavelengths, two values for the griseofulvin titre were obtained from the following formulae.

$$(i) \quad \frac{E_{288.0} - E_{261.5}}{0.0529} \times D = \mu\text{g/ml. griseofulvin in sample.}$$

$$(ii) \quad \frac{E_{288.0} - E_{322.5}}{0.9450} \times D = \mu\text{g/ml. griseofulvin in sample.}$$

E = absorption reading.

D = dilution of sample.

The two values given by these formulae should be considered separately as they are in fact two different estimates arrived at by using different portions of the



ultra-violet absorption curve for griseofulvin.

On carrying out this experiment it was found that the evaporation from the vials was appreciable and whereas by the first harvest on the fifth day only about 1.0 ml. was lost, at the final harvest (12th day) about 2.5 ml. of distilled water was required to bring some vials back to the original volume. The growth of the mould in these small fermentation vessels varied considerably between the synthetic media and the organic corn-steep liquor medium. The latter produced the more vigorous growth. The mycelium in all the media was in the form of pellets, the size of these being about the same in all vials.

The titres obtained in this experiment are given in $\mu\text{g.}/\text{ml.}$ in Table VII.

Table VII.

Examination of the results of this fermentation showed that the Czapek-Dox medium gave very inconsistent results at all the harvest times and from this aspect alone would be most unsuitable for a submerged fermentation in this type of vessel. That comparatively high titres can be obtained in vials with this medium was shown by the performance of some vials but these optimum conditions must be somewhat critical, bearing in mind that the same batch of medium was naturally used for all flasks of the Czapek-Dox group, and that the same inoculum was used throughout the experiment. The peak titre did not appear to occur with this medium before the final harvest, suggesting that a longer

TABLE VII

Trial fermentation of Strain I. in vials using
three media.

Titres in $\mu\text{g./ml.}$ to the nearest whole number.

MEDIUM	No.	5 days		7 days		9 days		12 days	
Czapek- Dox peptone	1	133	122	4	2	1	11	32	35
	2	35	38	8	7	11	7	88	72
	3	31	26	5	11	151	140	21	10
	4	37	41	41	51	9	15	120	107
	5	3	6	61	60	102	84	101	87
	6	39	57	39	45	63	50	89	74
	7	contaminated		36	30	57	52	66	62
Nitro- chalk.	1	136	90	93	79	128	118	116	95
	2	110	87	103	88	131	113	159	132
	3	127	142	107	91	143	145	152	137
	4	93	129	69	53	196	165	106	90
	5	90	78	88	75	149	152	112	91
	6	75	80	123	107	172	156	146	120
	7	86	61	60	38	167	149	148	120
C.S.L.	1	22	23	33	31	60	57	119	97
	2	5	7	41	43	112	93	96	83
	3	6	7	93	99	111	101	133	111
	4	7	9	126	109	73	78	151	128
	5	14	18	77	67	74	66	107	85
	6	9	9	24	23	86	98	114	95
	7	7	10	139	101	165	142	117	96

fermentation time would be necessary if this medium were chosen for the primary screen.

The results on C.S.L. medium, while they were more consistent at the two later harvest times, tended to vary considerably during the early part of the fermentation. It was not clear from the results whether the peak titre had been reached by the final harvest time but it appears likely that in general the titre was still rising at this time.

The nitro-chalk medium appeared to offer advantages over both the others ~~two~~. In addition to giving the most consistent results, the fermentation progressed more quickly in this medium and relatively high results were obtained as early as the fifth day. The peak titre occurred after about 9 days, which, if it was decided to use the peak as the harvest time for the primary screen, would allow a shorter fermentation than either of the other media. Taking all these facts into consideration, the nitro-chalk medium was the most suitable for use in the primary screen. It was also decided that this fermentation should be carried out for 7 days before harvest. This time was eventually chosen because it was envisaged that two types of mutants might be produced which would be satisfactory from the point of view of this programme. In addition to the mutant whose peak titre was higher than that of the parent and therefore would be capable of producing more griseofulvin in the same period of fermentation time, an organism might be isolated, which produced the same

amount of the antibiotic as the parent but achieved this in a shorter fermentation. From the indications given in the early experiments on the length of the griseofulvin fermentation, and which were confirmed in the experiment just described, it seemed likely that the fermentation would be a relatively long one, requiring about two weeks to reach completion (peak titre). Any mutant that could bring about a shortening of the fermentation would be a satisfactory isolate and a screening programme that ~~was~~ harvested in advance of the time of peak titre would tend to show up both these types.

Mutagenic Treatment.

Both ultra-violet and sulphur-35 treatment were used for the mutation of Strain I. The former treatment was to follow the lines of Farrell's (1953) sequential method which had been successfully used for the mutation of P. chrysogenum.

An experiment was performed to find out the killing rate of ultra-violet light, used under a certain set of conditions, on Strain I. The lamp used for this purpose was the same as that described in Part I. An agar-slope culture of Strain I. was prepared and grown up until it sporulated freely and from this a spore suspension was prepared by adding 10 ml. of sterile saline to the culture tube and gently scraping off the spores with a wire loop. This resulted in a very dense suspension and so it was diluted 10 times in saline. The diluted suspension was drawn in and out

vigorously through a Pasteur pipette for about ten minutes to break up the spore chains and a count was made using a haemocytometer. From this count ^{it} was calculated how much more dilution was necessary to give a suspension of approximately 1.0×10^6 spores per ml. When this further dilution had been carried out a second haemocytometer count showed that the suspension in fact contained 1.15×10^6 spores/ml.

One ml. of this suspension was removed and further diluted to serve as an untreated control sample. The remainder was transferred to a Petri dish and irradiated (with the top of the dish removed) with ultra-violet light for 5 minutes at a distance of 30 cm. from the lamp. During the irradiation the dish containing the spore suspension was gently agitated so that the spores situated at the edge of the dish should not remain in the same position throughout the treatment and thus possibly receive less treatment than those in the centre. At the end of each minute of treatment, the dish was removed from under the lamp and 1.0 ml. of the suspension was taken out and set aside; these samples received no further irradiation. At the end of the treatment the various samples including the control were suitably diluted and portions of each sample, calculated to contain a known number of spores, were transferred to C.S.L.-agar in Petri dishes and the spores spread with a glass spreader. Ten such dishes were prepared from each sample and incubated at 24°C . for 2-3 days by which time the viable spores germinated and formed colonies

that were large enough to count with the naked eye but which had not grown enough to sporulate and give rise to other colonies which would invalidate the count. The colonies from the untreated control sample were counted and the viability of the original suspension calculated. Taking this viability into consideration, the counts from the other samples gave the killing rates achieved at the time of the removal of the sample. The results of this experiment were as follows:

<u>Irradiation time.</u>	<u>%age kill.</u>
0 min. (control)	0
1 min.	64
2 min.	89
3 min.	98.8
4 min.	99.9
5 min.	>99.9

The viability of the untreated spore suspension was 77%.

Mutation rates given by sulphur-35 and ultra-violet treatment of Strain 1.

Experiments were carried out to discover the mutation rates that could be expected from the two mutagenic agents when they were used in the treatment of Strain 1. An ultra-violet irradiation was done on a spore suspension of this strain for 4 minutes, the procedure being the same as that described above. When the colonies had grown up on C.S.L.-agar, they were counted and the kill estimated at 99.8%. Fragments of mycelium from the edge of 400 of these colonies (selected at random) were transferred to minimal-agar medium and incubated for 7

days before the nutritional mutants were counted. In this experiment no nutritional mutants were found. The experiment was repeated again in case this result was abnormal but again no nutritional mutants were produced. This was unexpected, as the nutritional mutation rate achieved in these laboratories using the same technique on P. chrysogenum was between 0.5 and 0.9%. One explanation, which may account for this absence of biochemical mutants, is that the conidia of this strain may contain more than one complement of chromosomes. As far as can be ascertained, the literature does not contain any reference to genetical experiments having been carried out on P. patulum, but the cytology of certain other species of the genus Penicillium have been studied. Gueguen (1898-1899), who studied the cytology of P. glaucum and the formation of conidia in that species reported that there was only one nucleus in the conidium when separation from the sterigma was complete. More recently Baker (1944 a,b), studying P. notatum reported that conidia were usually uninucleate occasionally bi-nucleate. It seems unlikely that more than one nucleus is present in the conidia of this strain but the possibility that it is diploid must remain, as diploidisation is a frequent occurrence in nature. Verification or otherwise of this fact can now be carried out by the new techniques developed by Pontecorvo, Roper and Forbes (1953).

In view of this lack of nutritional mutants the colonies were grown to a greater size and the number of

morphological mutants among them counted. It was found that 15 of this type were obtained (3.75%), while in the untreated control sample two isolates out of the 400 were found to be morphological mutants (0.5%). It was realised that this selection of morphological mutants was open to subjective influences but as it was only intended to obtain a rough guide to the mutation rate produced by the two mutagenic agents, it was decided to adopt this method.

A sample of the same spore suspension as was used for the ultra-violet irradiation, was used for the sulphur-35 treatment. This was carried out as described in Part I. Strain 1, was found not to sporulate at all on the sulphur-free minimal medium containing only the radioactive isotope. In order to induce sporulation on this medium it was found necessary to add a small quantity of non-radioactive sulphate. An experiment carried out with different levels of sulphate added to the medium, showed that if about 1/60 of the concentration of sulphate present in normal minimal medium was added (in the form of sodium sulphate) to the sulphur-free medium, the mould would sporulate sufficiently to yield a workable spore suspension. A quantity of this medium was prepared and the sulphur-35 treatment was carried out. A treated and a control flask were harvested after 20 days and the resulting spore suspensions, after suitable dilution, were spread on C.S.L. agar in Petri dishes. 400 colonies from each of these treatments were picked off at random and tested for

nutritional mutants.

As in the case of the ultra-violet treatment, no biochemical mutants were found and the number of morphological mutants was counted as before. Among the treated colonies 129 morphological mutants were found together with seven doubtful ones about which a decision was difficult. This represented a mutation rate of between 32 and 34%, depending on the inclusion of the doubtful isolates. The untreated colonies yielded on inspection one certain morphological mutant and two doubtful ones (0.25-0.75%).

The criteria used in the identification and selection of these morphological mutants were those which could be most readily distinguished for example:

- (i) Considerable variation in pigment on minimal medium,
- (ii) Differences in texture of the surface of the growing colony, (iii) Marked differences in the colour of the conidia, as well as those having yellow or white conidia.

Although the selection of morphological mutants by this system was open to criticism, the selection was made by the same individual and it is considered that this method has some value as a comparison of the mutation rates. It was quite clear that the mutation rate produced by the 20-day sulphur treatment was greater than that produced by the ultra-violet treatment under the conditions used.

The technique adopted for the primary screening of mutants.

(a) Ultra-violet irradiation. A spore suspension

of Strain 1. was prepared in saline and diluted so as to give approximately 1.0×10^6 spores/ml. A 10 ml. sample of this suspension was irradiated for four minutes with ultra-violet light under the conditions described above and afterwards grown up on C.S.L. medium. A number of the viable colonies ~~were~~^{was} isolated on to C.S.L.-agar slopes and incubated. After they had sporulated they were stored in the cold until required. The same procedure was adopted for all the serial irradiations which were carried out.

(b) Sulphur-35 treatment. Further samples of the same spore suspension that was used for the first ultra-violet irradiation were used to inoculate the flasks containing the radioactive isotope. These flasks, containing 1.0 ml. of sulphur-free minimal medium (augmented with the extra sulphate) and 0.2 millicuries of sulphur-35 were incubated at 24°C . for 12 days, after which it was decided that growth and sporulation had ceased and they were stored in the cold until harvested. One flask was harvested after 20, 40, and 62 days respectively. In each case a spore suspension was prepared and after suitable dilution a portion of it was spread on C.S.L.-agar. After incubation a number of colonies were sub-cultured on to slopes of the same medium and grown up until an adequate supply of spores was available and then stored in the cold.

(c) The primary screen fermentation. Isolates derived from both mutagenic treatments were fermented under exactly the same conditions. A loopful of

spores was scraped off the surface of the culture and transferred to a sterile 30 ml. vial containing 7.5 ml. of nitro-chalk medium. Although a loopful of spores was a very arbitrary quantity to use, it was found to be satisfactory. With the relatively small quantity of medium present it appeared that only a small proportion of the spores transferred was needed to form a satisfactory inoculum and that the majority did not germinate. This fact was ascertained by microscopical examination of the broth from a sample of the vials from the first batch. The use of a loopful of spores implied the addition of excess material and was a satisfactory method of carrying out the inoculation. For convenience, the vials were inoculated in batches of 36, consisting of 30 isolates from the mutagenic treatment and 6 vials of Strain 1. to serve as controls. It was necessary to have this number of controls because, as yet, comparatively little was known about the griseofulvin fermentation and as can be seen from Table VII the seven-day titres of Strain 1, in nitro-chalk medium were subject to some degree of variation and it was felt that six control fermentations to each batch would give adequate information on the degree of variation inherent in the fermentation as it was being carried out. The vials were transferred after inoculation to a 2-inch throw rotary shaker and incubated for 7 days at 24°C.

While the mutant screening programme was in progress, as a result of other research being undertaken

in these laboratories, a production medium containing an organic source of nitrogen for the large-scale manufacture of griseofulvin was decided upon. In view of this, and of the desirability of ~~a~~ screening mutants on the same type of medium in which they would subsequently be fermented (this matter has been discussed in Part I, page 38) the fermentation medium for the primary screen was changed at a convenient time. This is explained more fully in describing the results obtained in the primary screen.

(d) Methods of assay. After shaking for seven days the vials were removed from the shaker and the volume of the medium lost by evaporation during the fermentation was made up with distilled water. The broth from each vial was transferred to a large boiling tube and extracted with cyclohexane and assayed as described in detail earlier (page 51). During the course of this programme it was found by Ashton that this method using cyclohexane as the extracting solvent was not extracting all the griseofulvin present in the broth samples. Ashton and Brown (in press) found that butyl acetate extracted a greater proportion of the total griseofulvin and gave a truer estimate of the titre. This information did not invalidate the work done in testing mutants so far carried out but it was decided to adopt this new extraction method as soon as possible. The butyl acetate assay method as described by Ashton did not lend itself to a routine assay for the primary screen and consequently it had to be modified in the following way.

After the removal of the vials from the shaker and the addition of the water to bring the volume of the medium back to the initial 7.5 ml. the broth from each vial was transferred to a boiling tube and to each of these tubes was added 15 ml. of butyl acetate (twice the broth volume). The tubes were corked tightly and then they were shaken manually for two minutes. In order to minimise the variation due to the differences in individual shaking methods, two tubes were shaken at a time (one in each hand) the tubes being held upright and they were shaken to a predetermined rhythm. These precautions ensured that each pair of tubes received the same amount of shaking. Attempts were made to shake the tubes mechanically on a reciprocal shaker but this was found to be much less effective. Owing to the speed of the machine much longer shaking was necessary. Small experiments were done using the same fermentation broth and the same volume of solvent to find out the minimum amount of shaking required to ensure maximum extraction of the antibiotic. From these tests it was evident that, using 7.5 ml. of broth and twice the volume of solvent, the maximum degree of extraction was reached after shaking for about $1\frac{1}{2}$ minutes. Thus two minutes was adopted to give a reasonable margin of excess.

After extraction the tubes were allowed to stand for about 30 minutes for the broth and the solvent to separate, after which 10 ml. of the solvent layer was transferred by pipette to other tubes and read on the

ultra-violet spectrophotometer in 1 cm. silica cells against a butyl acetate blank. Two readings only were taken in this assay, at 289.0 and 322.5 $m\mu$ and the titre was obtained by calculation from the following formula. (Ashton and Brown, in press).

$$\frac{E_{289.0} - E_{322.5}}{0.051} \times \frac{100}{5} \times D = \mu\text{g./ml. griseofulvin.}$$

E = absorption reading. D = dilution of sample.

The most accurate values from this assay are obtained when the butyl acetate sample read on the ultra-violet spectrophotometer contains between 5 and 20 $\mu\text{g./ml.}$ of griseofulvin (Ashton verbal communication), therefore care had to be taken in the dilution of the assay samples. In practice this method was found to work well and to be very much more accurate than the cyclohexane assay.

(e) Assessment of mutants. In the case of the ultra-violet isolates, Farrell's method was followed. This method did not involve any analysis of results. In the case of isolates derived from the sulphur-35 treatments, the titres were plotted in the form of a distribution curve and the controls also in a separate curve. It was hoped that this would give sufficient evidence from which to identify any mutants that showed improved yield merely by inspection. At the same time it would probably show whether there had been any considerable number of isolates produced whose capacity for producing griseofulvin had been reduced. In fact distribution curves were also prepared for the ultra-violet isolates.

The cyclohexane assay, which was used initially, gave two independent evaluations of titre. These values were averaged for the purposes of the distribution curve, so that each strain was represented by one plot on the graph. This spectrophotometric assay depends on the absorption curve of pure griseofulvin in cyclohexane. This curve shows a maximum at 288.0 m μ and two minima at 261.5 and 322.5 m μ , or more simply a peak with a trough situated on either side, the titre being given by the height that the peak is above each trough, hence the two values for the titre. It was envisaged that in the fermentation of a mutant, one or more substances might be produced by the mould, which were soluble in cyclohexane and whose ultra-violet absorption curve would interfere at one of the three points mentioned. This eventuality would result in the two estimates of titre given by the assay being widely different instead of close together as was normal. Such a mutant or series of mutants would be recognisable in the assay by the divergence of the two estimates but they would not be recognisable in the distribution curve where they would be represented by the mean of the two values.

Results obtained from the primary screen.

(a) First ultra-violet irradiation and 20-day sulphur-35 treatment. The results obtained after screening the first batch of ultra-violet treated strains (64 in all) and the sulphur-35 isolates obtained by treating the spores with the radioactive

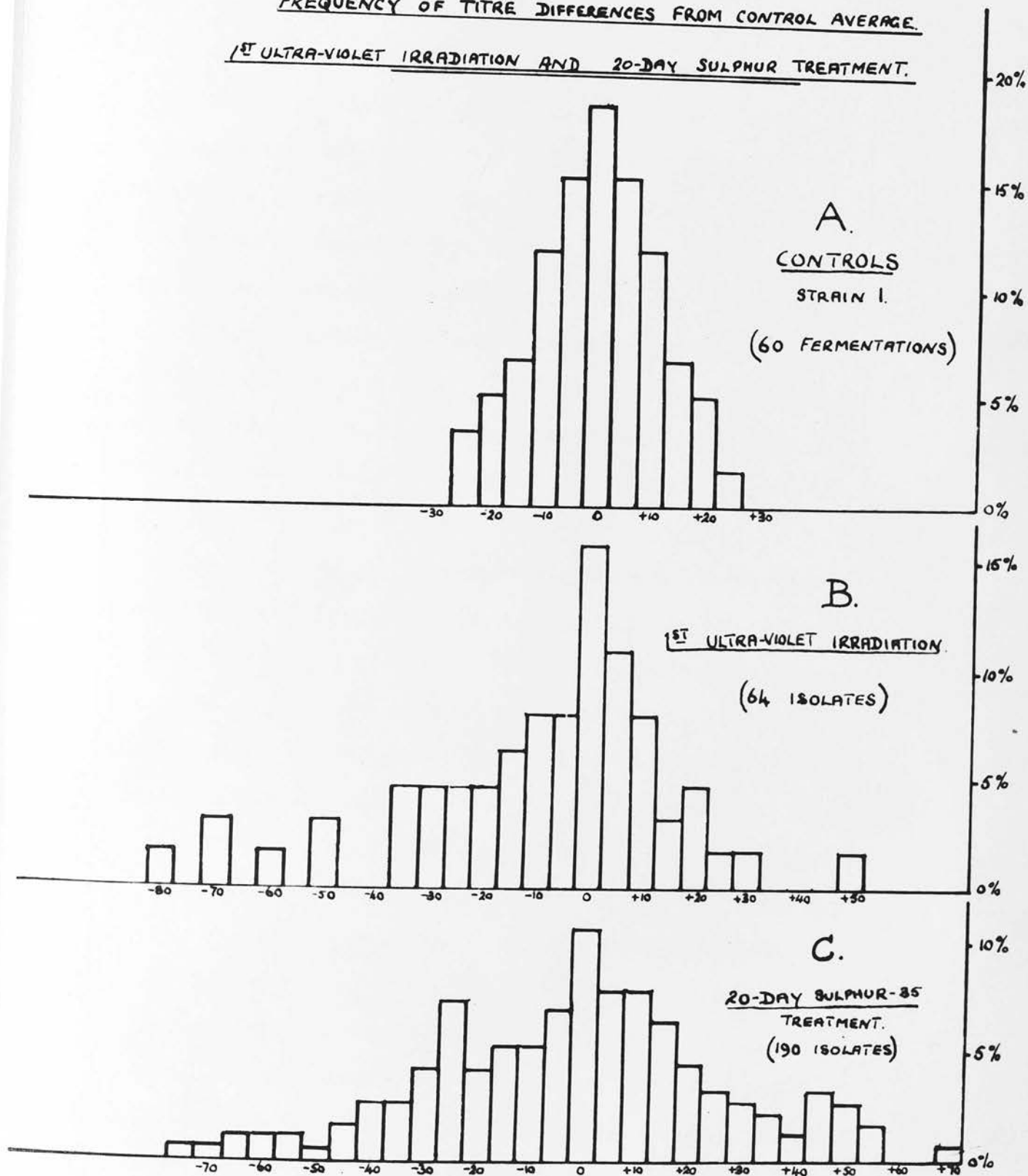
isotope for 20 days (190 strains) together with the controls for both these treatments are given in Fig. 3 in the form of histograms. The ultra-violet isolates were tested in two batches requiring only 12 control fermentations and it was not considered worth while preparing a separate histogram for such a small sample of control fermentations, consequently they were combined with the 48 control fermentations carried out during the testing of the 20-day sulphur-35 isolates. This is a legitimate amalgamation as all the control fermentations were conducted under identical conditions.

In the preparation of these histograms, in order to eliminate possible variation in the fermentation conditions from experiment to experiment, putative mutants were compared with the controls fermented at the same time. All results of controls and mutants were expressed as the difference in titre ($\mu\text{g/ml.}$) between individual fermentations being considered and the average of the controls in the same batch. The results obtained by the ultra-violet and sulphur-35 treatment (histograms B and C respectively) are compared graphically with the untreated controls (histogram A). The frequency with which any given difference from the average control was observed is plotted as a percentage against the magnitude of the difference, though for convenience the observations were grouped in ranges of $5 \mu\text{g/ml.}$; thus differences of from -2.5 to +2.49 were called zero and +2.5 to +7.49 were called +5 and so on.

The resulting histogram for the controls (A) shows

Fig 3

FREQUENCY OF TITRE DIFFERENCES FROM CONTROL AVERAGE.
1ST ULTRA-VIOLET IRRADIATION AND 20-DAY SULPHUR TREATMENT.



a straightforward symmetrical distribution such as would be anticipated if the fermentations were subject to normal error distribution. In the case of the ultra-violet and sulphur-35 treated strains it is quite clear that appreciable numbers are affected, since if no alteration had taken place they would be expected to follow the normal error distribution as for the controls. Since each fermentation is derived from a single spore isolate and since the yields obtained are derived from a very large number of mycelial cells not themselves treated, it follows that the alteration in the original spore has been transferred to the mycelium as a whole, a situation scarcely explicable by any other means than mutation.

The most obvious distinction between ultra-violet and sulphur-35 treatment is that the former, as is common with mutagenic agents, tends to produce an excess of poor-titre mutants with only very few high-titre strains; while the latter treatment appears to produce high and low-titre mutants with much less bias. Strain 1. is a wild-type strain not previously subjected to artificial mutation and because of this does not show the tendency (as did P. chrysogenum Strain A. see Part I) to revert to an earlier stage of its development. In fact it would not be unreasonable to postulate a continuous spectrum of mutants, the frequency of their occurrence being such as to yield a very flat graph. This, superimposed on the symmetrical distribution resulting from those strains not actually affected by the sulphur-35,

would yield a graph very similar to that observed. Several theories have occurred to the author to account for this difference in the results of the two mutagenic treatments. It was felt, however, that a series of different treatments of both ultra-violet and sulphur-35 would have to be studied before any useful postulation could be made. It is of significance that the results given ~~are~~^{are} derived from treatment under one set of conditions only for each mutagenic agent.

(b) Testing of morphological mutants. In addition to the 64 ultra-violet isolates already mentioned, an extra batch of ultra-violet isolates ~~were~~^{was} tested on the primary screen. This was composed of the 15 morphological mutants mentioned earlier as having been obtained while finding out the mutation rate given by the ultra-violet treatment, together with the same number of strains from the same source, which, although they were not sufficiently different to be definitely classified as morphological mutants, had shown some differences from the control. This collection of isolates was roughly classified according to the type of difference they displayed (i.e. more pigment produced than the control, less pigment, darker spore colour, etc.). This was done to ascertain whether there was any correlation between morphological variation and antibiotic production. The titres obtained from these strains were not incorporated in the histogram because, being almost certainly mutants of a type, they were a special selection and therefore

different from the other isolates which were a random selection of treated spores. The results of this test showed that there was very little alteration of titre among these strains; two gave titres of below 40 $\mu\text{g/ml.}$ and one above 100 $\mu\text{g/ml.}$, the remainder were distributed fairly evenly about the control. This test tended to confirm what many workers have reported when dealing with other species - namely, that there is no obvious correlation between a particular type of morphological mutant and alteration in titre of metabolic products.

A similar test was carried out with morphological mutants obtained from the 20-day sulphur treatment. Although in this instance there was rather more alteration in titre than in the previous test no correlation between mutant type and titre change could be observed.

(c) High-titre strains produced. One isolate derived from the ultra-violet treatment (No.1/2) gave a titre of 162, 166 $\mu\text{g/ml.}$ in the primary screen fermentation against a control average of 112 $\mu\text{g/ml.}$ (an unusually high control titre average). This isolate represented an increase of about 47% and it was chosen for the second irradiation.

Of the 190 isolates derived from the 20-day sulphur-35 treatment two were chosen as being outstanding as follows:-

(i) S.48 gave titre of 142, 141 $\mu\text{g/ml.}$ against control average of 71 $\mu\text{g/ml.}$

(ii) S.152 gave titre of 132, 167 $\mu\text{g/ml.}$ against control average of 77 $\mu\text{g/ml.}$

As can be seen from the histogram (C) there ~~were~~^{was} a number of strains around the +50 mark but the two mentioned above were chosen for further testing because they gave the highest increase over the average control. The isolate S.152 represented approximately 100% increase in titre over the parent (Strain 1).

(d) The second ultra-violet irradiation and the 40-day sulphur-35 treatment. The second irradiation by ultra-violet light was conducted in exactly the same way as the first. It was carried out on isolate No. 2/12, the highest yielding strain produced by the first treatment. On this occasion, however, 170 viable colonies were isolated for testing on the primary screen, in order to provide a larger sample for the study of titre distribution. It was felt that the 64 strains tested from the first irradiation were only just sufficient to give a satisfactory graph with the titres spread as they were over a wide range. These 170 strains were tested on the primary screen against the parent (No. 2/12) as control.

The culture of Strain 1. harvested 40 days after inoculation on the sulphur-35 medium was treated in the same way as the 20-day culture and 220 colonies were isolated for the primary screen. The appearance of this particular flask at the time of harvest differed somewhat from the remainder (to be used for later harvests) in that it had sporulated rather more vigorously. This observation was confirmed by the haemocytometer count on the resulting suspension which

was 8.3×10^6 spores/ml. as against counts of between 2.0 and 4.0×10^6 spores/ml. for the other flasks. Serious consideration was given as to whether this flask should be used or one which appeared to have more normal sporulation. As, however, the flasks had been randomised after inoculation and each flask identified with a harvest time it was finally decided to use this flask to provide the spores treated for 40 days.

Isolates from these two treatments were tested on the primary screen and assayed as before by the cyclohexane method. Histograms were prepared showing the frequency with which the titres differed from the average control titre of the batch. These histograms are given in Figs. 4 a & 4 b. (D, E, F and G). In this case the controls for the ultra-violet and sulphur-35 isolates were not amalgamated in one graph because they were not the same strain. This has been explained above.

As can be seen from histograms D and F, the controls both followed the normal error type of distribution. Histogram E, however, differs noticeably from that of the first ultra-violet irradiation in that the spread of titres obtained is greater on the side showing improved titres and moreover the overall mutation rate is higher. The resulting distribution graph (E) is very flat and is much nearer the shape of the curve obtained from the 20-day sulphur isolates than it is to the curve of the first ultra-violet irradiation. This phenomenon is discussed in the general discussion.

FIG 4A

FREQUENCY OF TITRE DIFFERENCES FROM CONTROL AVERAGE.

2ND ULTRA-VIOLET IRRADIATION.

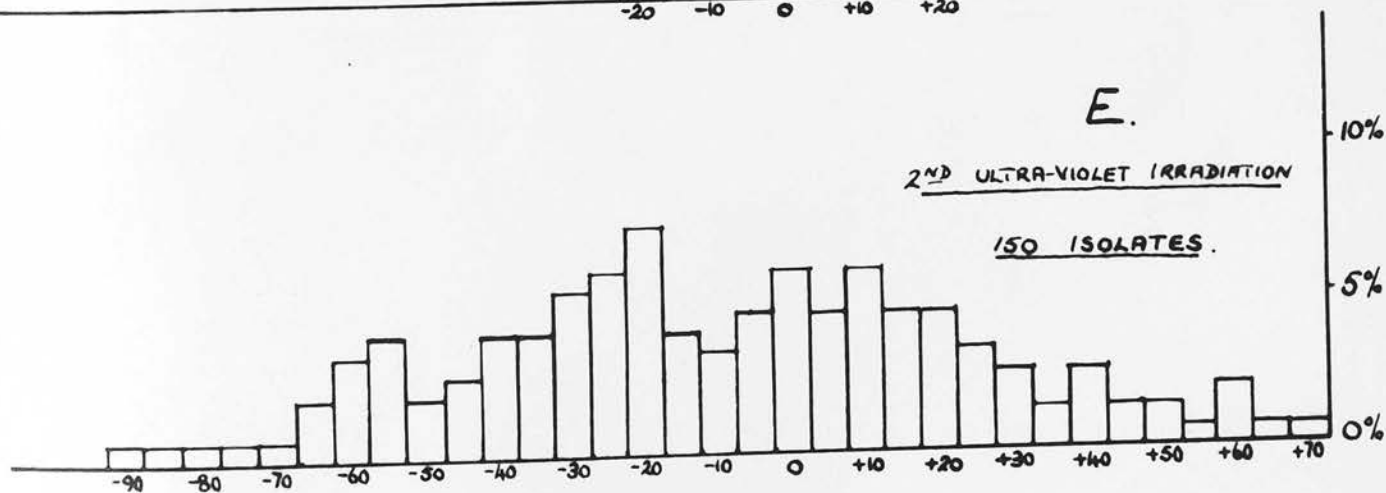
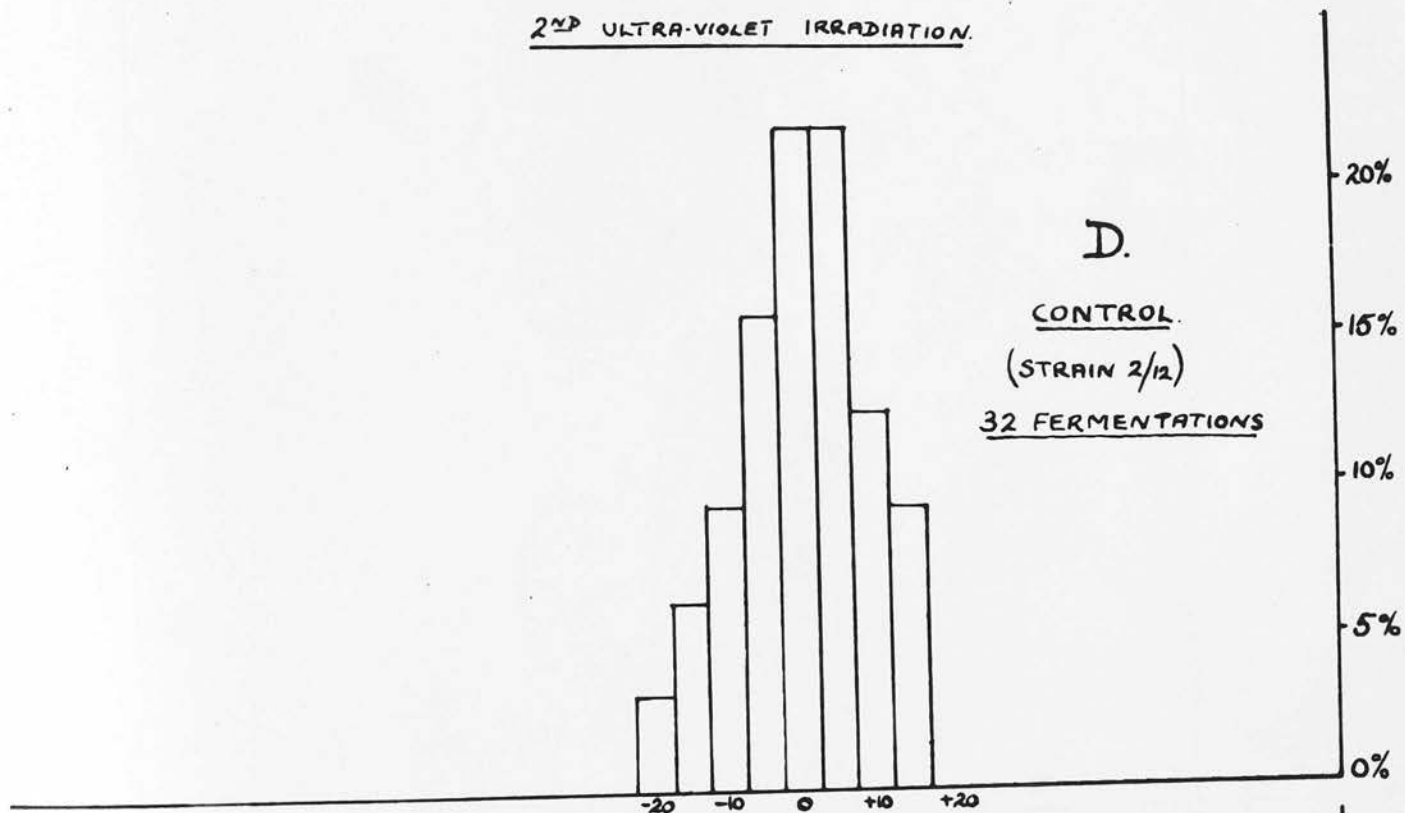
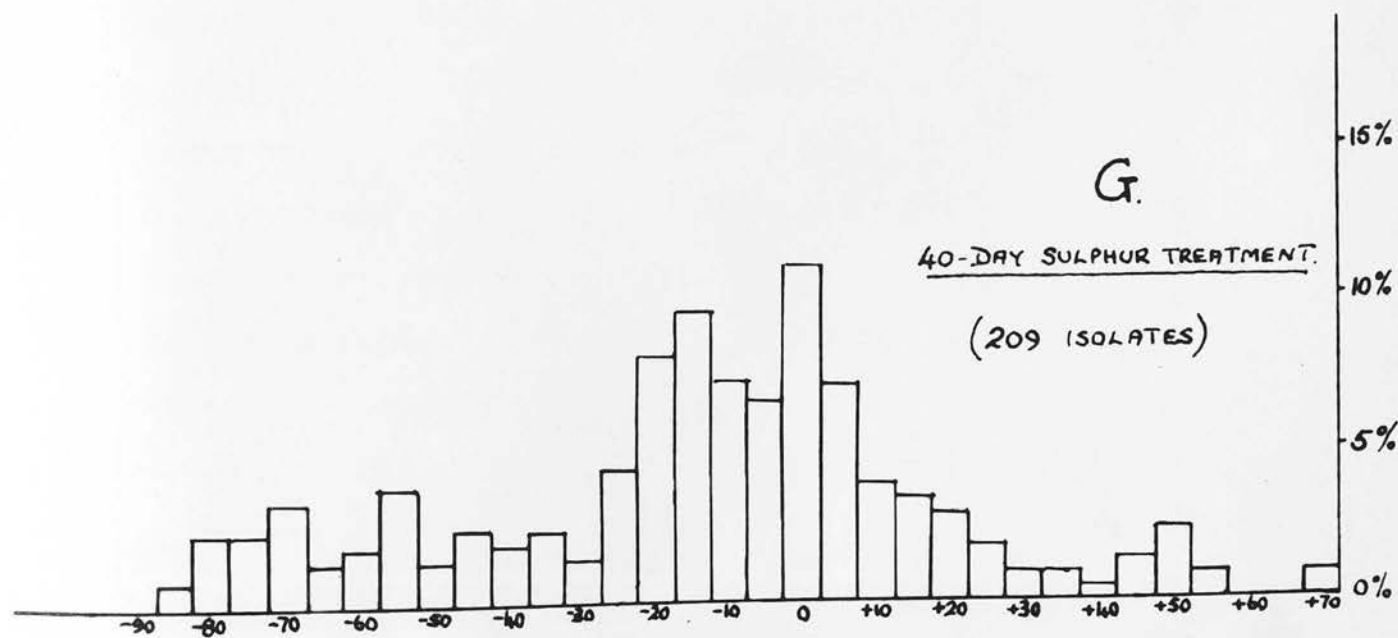
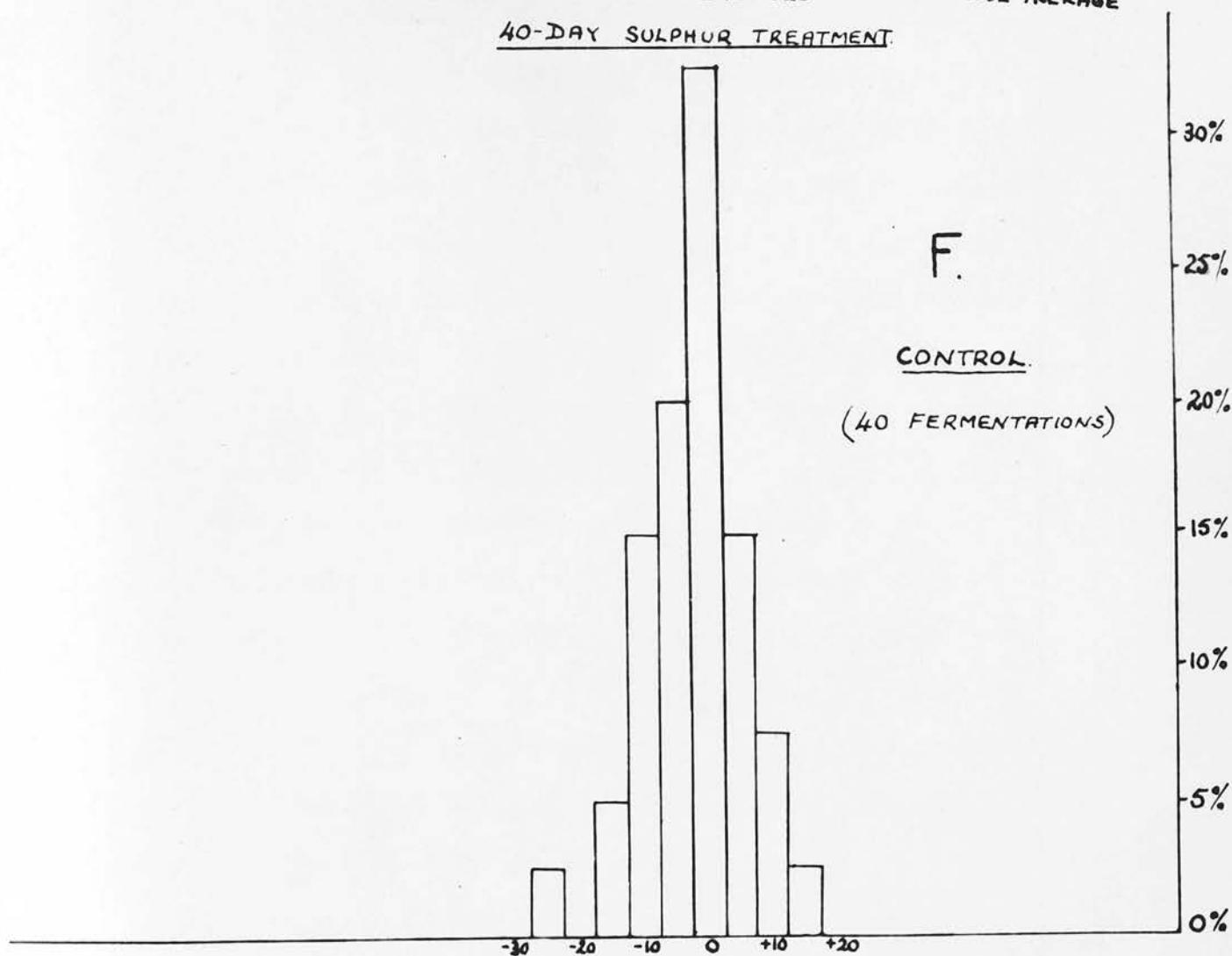


FIG 4B

FREQUENCY OF TITRE DIFFERENCES FROM CONTROL AVERAGE

40-DAY SULPHUR TREATMENT.



In the case of histogram G. showing the distribution of the 40-day sulphur isolates, the mutation rate does not appear to have increased materially with the extra time of treatment, apart from a marked build-up in the region of -70 to -80. According to the theory of the action of sulphur-35 as a mutagenic agent (see Part I, page 26), the mutation rate depends, among other factors, on the number of sulphur-35 atoms that have changed to chlorine-35, that is to say on the length of time which has elapsed since the mould started to grow in contact with the isotope. Thus one would expect a greater mutation rate after 40 days than after 20 days. This has been shown to be the case with P. chrysogenum and there is no reason to suppose that it should be different with P. patulum. It seems more likely that the reason is indicated by the sporulation difference noted above. As mentioned earlier, a small quantity of sulphur-32 was added to the culture medium to bring the total sulphur content up to a level which would allow reasonable sporulation. Although great care was taken to ensure that the same amount was added to each flask it is possible that some unevenness of distribution did occur in this flask. From this the histograms G and C are not strictly comparable with regard to mutation rates but it is interesting to compare these two graphs in another respect. It may be noticed that at the following points on graphs G and C viz. +70, +45 to +55, -15 to -25, and -55 small aggregates of mutants occurred whose titres differ from

the control average by about the same amount. ~~Of these aggregates~~ There are signs of similar groups among the ultra-violet irradiated strains but aggregations at other points are not obviously duplicated. The possible significance of these aggregates and their duplication or non-duplication is discussed at greater length in the general discussion at the end of this thesis.

In histogram **G** an aggregation is evident at the minus 70-80 level which is not repeated on any of the other graphs. These isolates are made up of ~~these~~ organisms giving virtually no titre at all; the average control titres varied between 70 and 90 $\mu\text{g/ml}$. A few isolates among the 20-day treated sulphur strains fell within these limits but their frequency had increased considerably after the 40-day treatment. These strains probably represent spores which remained viable after a double mutation, as in other respects the mutation rate does not seem to have increased.

The accurate testing of promising strains indicated by the primary screen.

At this stage it was thought advisable to test those strains, which had been indicated by the primary screen as being potentially higher griseofulvin producers, on a medium suitable for large-scale fermentation developed by other workers in these laboratories, to find out if their performance would be repeated on a different type of medium.

The following strains were selected from the

various mutagenic treatments already done. They were chosen because they showed the greatest improvement in titre over the parent strain. Details of their performance on the primary screen are given below.

<u>Strain No.</u>	<u>Mutagenic treatment</u>	<u>Titre.</u>	<u>Control titre average of batch.</u>
S.48	20-day S-35	142, 141	71
S.152	20-day S-35	132, 167	77
S.216	40-day S-35	130, 139	67
S.200	40-day S-35	150, 154	95
S.294	40-day S-35	132, 158	90
S.318	40-day S-35	132, 161	90
S.369	40-day S-35	146, 154	80

} Tested in
same batch.

The isolate showing the highest titre in each ultra-violet irradiation (No. 2/12 from the first and No. 3/6 from the second) was also included in the accurate retest, making ten strains in all, including the control. The performance of these two ultra-violet isolates on the primary screen ^{is} ~~are~~ given below.

<u>Strain No.</u>	<u>Titre.</u>	<u>Control titre average of batch.</u>
No. 2/12	162, 166	122 (an unusually high average)
No. 3/6	153, 185	95

Ten 250 ml. conical flasks containing 60 ml. of a suitable development medium were inoculated with spores of the above isolates and shaken for 48 hours at 24°C. This provided a thick vegetative inoculum for the fermentation flasks. Two flasks, each containing 60 ml. of the production medium, were inoculated with each strain and the control (20 flasks in all) and shaken at 24°C for 13 days. Three strains and the control were

fermented on until the 18th day. After 5, 7, 10 and 13 days, 3 ml. of broth was aseptically removed from each flask and assayed individually, after butyl acetate extraction in the method already described. These results are given in Table VIII.

At the conclusion of this test the control flasks and those of isolate S.152 were handed over to the chemical assay laboratory in order to make certain that the manual shaking technique was in fact extracting the maximum amount of griseofulvin, and to see if any dechlorogriseofulvin had been produced in the fermentation. Although up till now Strain 1 had never produced any dechlorogriseofulvin a mutant of this strain might produce some. As a result of this total extraction test the titres on day 18 were confirmed and no trace of dechlorogriseofulvin was found in the fermentation broth.

The results obtained from this accurate retest are of considerable interest. In the first place only two of these mutants (S.152 and S.369) have produced the 100% increase in titre that they produced in the primary screen on nitro-chalk medium. A further four strains (S.216, S.200, S.294 and S.318), while yielding in excess of the control, only achieved an improvement of 30% or less. The remaining three isolates, including the two derived from ultra-violet treatment, produced the same or lower titres than the control. These results clearly bear out the fact, already stressed in Part I, that mutants selected for a characteristic

TABLE ~~IV~~VIIIAccurate retest of promising mutants in the
production medium.

(Titres are expressed as a percentage of the peak
control titre).

STRAIN	5 th day	7 th day	10 th day	13 th day	18 th day.
Strain I (control)	18.46	42.5	100.	95.55	80.9
S.48	18.1	28.6	63.8	71.4	
S.152	43.4	94.0	136.8	169.5	199.8
S.216	24.3	53.6	127.8	118.4	
S.200	21.3	39.6	82.4	116.1	
S.294	23.1	50.8	119.4	130.0	110.5
S.318	23.2	55.6	116.5	121.5	
S.369	31.4	66.6	124.8	143.2	187.8
No.3/6	26.95	50.2	87.8	98.5	
No.2/12	20.66	34.5	67.2	83.6	

shown on one type of medium will not necessarily repeat this performance on another.

A further interesting fact is brought out by this accurate retest. As can be seen from the performance of these isolates in the primary screen, they were all about equally superior in griseofulvin production to the parent strain. One might have been tempted to infer from this that they had received closely related mutations. It is quite clear from this retest, however, that a number of widely differing mutations must have been induced in them.

The failure of the ultra-violet isolates to reproduce their high titres on the new medium, whereas the majority of those selected from the sulphur-35 treatment did show some improvement on the control, is a disturbing feature. It is evident that they were medium-specific mutants as was the sulphur strain S.48. Unfortunately it was not possible to carry out accurate retests on larger samples of the improved strains from both treatments, on the production medium. Such tests would have indicated whether the proportion of medium-specific mutants was greater from the one treatment than from the other. It is nevertheless an investigation which needs to be carried out and is probably one of the simplest ways of establishing any intrinsic differences in the mutagenic quality of the two treatments, if any do exist.

The lack of improvement, on the production medium, shown by the two chosen isolates from the ultra-violet

treatments, demonstrated clearly that in this part of the mutation programme a medium-specific mutation had been introduced, which would have to be eradicated before these mutants could be made use of on this medium. Because of this, and owing to the fact that a satisfactory griseofulvin producing strain had been isolated, it was decided to discontinue the ultra-violet part of the programme and to conclude this work by testing the sulphur-35 strains isolated after 62 days treatment. These remaining isolates were tested in order to complete the data on sulphur-35 treatment and the time effect. In addition it was done to find out if any mutants could be isolated whose performance on the production medium was superior to the two already mentioned. (S.152 and S.369). These two mutants were selected as being superior to the parent (Strain 1) on both types of media but it is possible that mutants may have been induced whose performance was not so good as these two on nitro-chalk medium but better on the production medium.

The two best sulphur-35 mutants were considered to be quite suitable for further development and testing on a larger scale. This process was not undertaken by the author and is outside the scope of this thesis. Before handing over the strains, however, they were examined morphologically in some detail to try and find if there were any differences between them and the parent.

Morphological examination of sulphur mutants (S.152 and S.369).

These two strains and the parent were grown up

together on a number of solid media and examined microscopically. The sporing surfaces of the growing colonies were examined in situ and preparations were made of the mycelium, penicilli and spores. It was evident from these comparisons that no discernible morphological differences had been induced by the mutations. A photograph of the three strains growing on malt agar was taken (Plate I,b) and the colonies (13 days old) agree closely with each other and with the type photograph given by Raper and Thom (1949 p.535).

The only differences that could be distinguished were of a physiological nature and they are outlined below.

(a) Pigment production. Strain 1, the parent, produces on minimal agar a reddish-brown pigment which is clearly visible on the underside of the colony and which diffuses into the medium and tints the agar out beyond the colony. S.369 produces less of this pigment and the underside of the colony is noticeably lighter in colour. In the case of S.152 no difference in pigmentation can be seen. No pigment is produced by any of these three strains on malt or Bennett's agar.

(b) Growth rate. The growth rates of the parent and the two mutants were compared at 25°C on malt, minimal and Bennett's agar (15 ml. of medium in a 9 cm. Petri dish). The diameters of the colonies were measured in mm. after 9 and 15 days. The results are given below, in tabular form. (The

figure for each strain is an average of 5 strains)

<u>Minimal agar.</u>	<u>9 days.</u>	<u>15 days.</u>
Strain 1	25.0 mm.	36.5 mm.
S.152	23.7	31.2
S.369	23.5	31.4
<u>Bennett's agar.</u>		
Strain 1	32.3	50.9
S.152	30.4	48.4
S.369	33.1	51.2
<u>Malt agar.</u>		
Strain 1	30.05	45.1
S.152	29.3	44.1
S.369	31.4	47.8

It is interesting to note from these results that whereas S.152 has a slightly slower rate of growth than the parent on all these media, S.369 on the other hand has a slower growth rate on the minimal medium but on the two richer media its growth is rather faster than the parent.

(c) Odour. Raper and Thom (1949) describe some isolates of P. patulum as having a distinctive and fragrant odour on Czapek-Dox agar. Strain 1 does not have this character. A panel of ten people were asked to cooperate in this part of the investigation and whereas they were unanimous on the lack of smell associated with Strain 1, half the panel considered that there was a very slight odour associated with S.152 and S.369, although they were reluctant to describe its nature other than to say it was not unpleasant.

(d) Sectoring. Up to the time of writing neither of the two mutants had shown any great tendency to produce sectors in culture as was the case with the two mutants of P. chrysogenum, see Part I. As can be seen from the photograph (Plate I, a) the parent ~~is~~ produced~~y~~ some faint sectors on malt agar. This ~~was~~ not unexpected as this strain ~~was~~ a wild type, which almost inevitably will be heterocaryotic for some genes.

The results of the primary screening of 62-day sulphur-35 isolates.

The remaining culture of Strain 1, growing on a medium containing radioactive sulphur-35 was harvested 62 days after its inoculation and from the resulting spore suspension some 600-700 isolates were made with a view to putting all these through the primary screen if necessary. These isolates were screened in the same way as the 20 and 40-day isolates, except that the medium used was the production medium. The butyl acetate extraction and assay was also introduced in the primary screen for the first time because it had been shown to be more accurate in the assessment of the griseofulvin content of the fermentation broth. Because of the change of medium and assay procedure, the titre distribution of these isolates can not be compared with that given for the earlier screenings and it is not given.

Some 520 of these 62-day isolates were actually put through this modified primary screen and out of these no strains were found which were better or even

as good as the two isolates already chosen for further development. This does not imply that there were no strains showing an improved titre over the parent strain. In fact there were many of these but the improvements found were ^{only} up to about 30% better than the control with one or two strains showing an increase in the region of 50%. A careful scrutiny of these results showed that there were a number of isolates giving virtually no griseofulvin and that these strains represented ^{ed} about 17% of the total number tested. It can be seen by referring back to the distribution graphs C and E, (the titre distribution graph of the shorter sulphur-35 treatments) that the percentage of mutants giving no griseofulvin in the primary screen was approximately 5% in the 20-day isolates and 10-11% in the 40-day isolates. The two earlier values were obtained on a nitro-chalk medium and the last on the production medium. There is no evidence which could justify the assumption that mutations causing loss of the ability to produce the antibiotic are any less likely to be medium specific than those causing partial loss or increase in griseofulvin production. Thus it would be dangerous to compare the 17% too closely with the 5 and 10-11% of earlier treatments. This does not contradict the theory that these non-producing strains may represent those which sustained double mutations, which it is reasonable to assume would increase in number with the increase in time of treatment.

An attempt was made to assess the titre mutation

rate shown by these 62-day isolates and it was evident that it was somewhat higher than both the two previous treatments, but in view of the modifications to the primary screen it was not considered profitable to make any close comparison.

Conclusion.

In considering this mutation programme, which has been undertaken on a griseofulvin-producing strain of P. patulum, it is necessary to examine first the experimental methods employed and suggest how they could be improved upon in the light of the experience gained.

The fermentation technique, using 30 ml. vials, proved effective and improved mutants have been detected from these fermentations. By using these vials instead of the more usual 250 ml. conical flasks it is estimated that a five-fold increase in the number of strains tested in a given time was achieved. This in itself is an advantage but against this must be placed the fact that the smaller vessel is definitely more liable to error in that smaller quantities of medium are used and inaccuracies in charging the vials are magnified. In a primary screening programme of this kind these errors were not great enough to invalidate the use of this vessel. Their manipulation and use did not provide any difficulties as compared with the space needed for the larger flasks. They are considered to be satisfactory for conducting primary screen type fermentations.

Of the assay methods used in the course of this work, that involving butyl acetate extraction is undoubtedly superior to the cyclohexane method which would not have been used had the former been developed when the programme was planned. The cyclohexane was in fact the method which indicated the two chosen mutants and it appears that although cyclohexane only extracts part of the griseofulvin available in the broth and mycelium, the amount extracted is proportional to the amount present and the method did give a true indication of those strains which were producing more of the antibiotic. In any future mutation programmes involving the griseofulvin yield of micro-organisms, butyl acetate or dimethylformamide (Ashton and Rhodes 1955) would be the solvent of choice.

The only biological assay which is known at the present time, namely that discovered by Brian, Curtis and Hemming (1946) is quite unsuitable for dealing with large numbers of routine samples. So long as one is quite certain that the substance being assayed is in fact griseofulvin the ultra-violet spectrophotometric assay is quite satisfactory. It must however be borne in mind that analogues of griseofulvin do exist, for example dechlorogriseofulvin, whose absorption curve is close to that of griseofulvin and should a mutant be isolated that synthesised a mixture of the two substances or the dechloro-analogue alone, then an investigation based solely on the ultra-violet absorption assay would be liable to give a false impression of the

fermentation titres. In such a case the biological assay would be needed in conjunction with the physico-chemical method. A simple biological assay would be extremely useful if such could be developed. This possibility is considered in the general discussion at the end of the thesis.

Sulphur-35 has been shown to be a potent mutagenic agent for manipulating the griseofulvin yield of P. patulum and the penicillin yield of P. chrysogenum. It must therefore be added to the growing list of treatments which have been used successfully to produce strains of micro-organisms giving increased yields of antibiotics. Up to the present the only other report on its use as a mutagenic agent is the original one by Hungate and Mannell (1952) in which it was used on Neurospora crassa.

GENERAL DISCUSSION.

It is in the nature of scientific research that the study of a problem very often brings to light many more questions than are answered by its solution. This research programme was planned to find out if a radioactive isotope, such as sulphur-35, is an efficient agent for mutating micro-organisms to achieve increased antibiotic yield, and if it is, how it compares with other agents already known and tried. Coupled with this main problem was a subsidiary one involving the improvement of methods of identifying any suitable mutants which might be produced.

Consideration of sulphur-35 as a mutagenic agent.

The answer to the first of these questions has been shown to be in the affirmative. In the case of the two fungal species studied, mutants have been produced whose antibiotic yield was significantly higher than that of the parent strain. In the first species (Penicillium chrysogenum) one improved mutant, showing a 28% increase in titre over the parent, was isolated from among 492 isolates screened. In a second mutation programme, using a selected variant of the first mutant as the parent, a second mutant was isolated which was capable of achieving the same titre as the first but on a slightly weaker medium. This mutant was isolated after some 800-900 isolates had been tested. In addition to these two, other strains were indicated by the primary screen as being capable of slightly better titres than the parent. It may be fortuitous that these strains were discovered after comparatively few putative mutants had been tested but the capability of this mutagenic agent for altering the penicillin yield of the species has been established. Strain A, the organism used as the parent in this work, had already been mutated at least four times, each mutation resulting in a substantial increase in penicillin production (Perlman 1950) (Backhus and Stauffer 1955). In addition to these four mutations at least one natural variant, probably more, have figured in the genealogy of this strain. Although no direct comparison between the efficiency of sulphur-35 and other treatments was possible in Part I of this work, a certain comparison

can be drawn with ultra-violet irradiation from previous work carried out by the author. As mentioned in Part I, just prior to the programme of research described in this thesis, an extensive mutation programme had been conducted involving the ultra-violet treatment of P. chrysogenum Strain A. to obtain improved penicillina yields. During this programme over 10,000 putative mutants were screened and among them no strain was found which gave significantly greater antibiotic yield when accurately retested. Backhus and Stauffer (1955) have shown that in their extensive mutation programme at Wisconsin using P. chrysogenum Q.176 they obtained improved mutants more easily using nitrogen mustard as a mutagen than with ultra-violet treatment. There is a possibility that in the development of the strain Q.176 and Strain A, this line of mutants has ~~been~~ had induced in it a genotype, such that ultra-violet treatment was less able to effect changes in antibiotic yield than were other methods of treatment.

In the case of Penicillium patulum, sulphur-35 treatment of the wild type Strain I has resulted in the isolation of two mutants that show about 100% improvement in titre on the parent. These mutants were obtained in the first 395 isolates screened. In addition to these, several other mutants whose improvement in griseofulvin yield was more or less medium-specific were obtained. A simultaneous programme involving ultra-violet treatment of Strain I, after the method of Farrell (1953), yielded no mutants that were not medium-specific out of 214 isolates tested in two

sequential irradiations. Mutants showing improved titres on the nitro-chalk medium were obtained.

Comparison of sulphur-35 and other mutagens.

The fact that sulphur-35 is assimilated by the organism and is used in the synthesis of some part of the gene complex, puts it in a different category from the more orthodox mutagenic agents, which induce mutation by bombardment or chemical attack on the gene from the outside. This does not mean that the mutations caused by it are necessarily different from those caused by other agents, indeed the indications are that the majority of the mutations induced by sulphur-35 are the same as those caused by other agents, for example mutants having yellow and white conidia. It may, however, by its very nature, suffer from one disadvantage, namely that if the organism possesses any genes which do not contain the element sulphur in a capacity essential to their normal working, these genes may be less liable to mutation by the radioactive isotope than those which do. That there is a possibility of their mutation by the weak beta-particle emitted when the atom disintegrates has been shown by Hungate and Mannell (1952). It is generally considered that the majority of genes do contain sulphur but the sulphur present may not, in all cases, be essential to the normal working of the genes.

Another important advantage held by sulphur-35 over ultra-violet irradiation and in fact over the majority of mutagenic agents, is the increased mutation

rates obtainable with it. In treating P.chrysogenum Strain A., an alteration was obtained in the penicillin yield of about 60% of the isolates. With P. patulum an alteration in the griseofulvin yield of between 40 and 50% of the isolates was observed. This is greater than anything so far reported in the literature dealing with the antibiotic yield of a micro-organism. The disadvantage of radio-active sulphur is that the treatment requires a number of days to carry out whereas other mutagenic treatments such as ultra-violet, X-rays or nitrogen mustard can be completed in a few hours.

Hungate and Mannell (1952) have claimed that after sulphur-35 treatment, mutant types of Neurospora crassa were isolated which had not been encountered after treatment with other mutagenic agents. With reference to this claim it is worth recording that during the mutation programme involving P. chrysogenum Strain A., morphological types of this organism were encountered which had not been seen before although a study of mutants of this strain obtained by using other mutagenic agents had been carried out by the author over a period of three years. As the study of these mutant types, other than of their penicillin yield, fell outside the scope of this work, it was not possible to study them in more detail but it is a line of research which would probably repay further study. If it could be definitely established that sulphur-35 treatment yielded mutant types which were not obtainable by other means it would be a discovery of great importance. No

observations on this question can be made regarding the morphological mutants of P. patulum isolated in the second part of this work because the author's experience with this species is more limited.

Aggregation of mutants in titre-groups.

An interesting feature of the mutants obtained from both the fungal species studied in this work was the fact that they tended to fall into groups producing roughly the same titre. This was more evident in the case of the sulphur-35 mutants than with the ultra-violet, presumably because of the higher mutation rates obtained with the former treatment, which made the fact more obvious. As far as can be ascertained there has been no mention of this tendency in the literature, to date. It is, however, a phenomenon which might have been expected. It is reasonable to suppose that in any micro-organism which produces an antibiotic there is a definite number of genes and their alleles which influence its synthesis. To carry the hypothesis one stage further there is also a finite number of gene combinations influencing the amount of the antibiotic produced. Under a fixed set of culture conditions the titre produced by one genotype (for example different subcultures of the same pure strain) are remarkably constant. This fact is known from fermentation experience. Genetical change induced by a mutagenic agent, if it affects a gene or genes which have no bearing on the synthesis of the product, directly or indirectly, should not alter the titre provided the conditions of

culture remain constant. If a mutation occurs in a gene or genes having an influence on the synthetic system in question it will form with the other genes so concerned a combination which has a definite "titre value" under constant culture conditions. It seems likely that the aggregates observed in the titre distribution graphs represent organisms whose genotypes have the same "titre values". If this explanation is correct, a study of the various mutants making up these aggregates could provide useful information as to how the synthesis of the antibiotic is controlled.

Is sulphur-35 a specific mutagen?

There exist in the literature at least two claims that certain chemical substances are specific for certain genes in their mutagenic effect. Gustafsson and MacKey (1948) pointed out that there was a different distribution of certain albino mutants in barley following nitrogen mustard treatment than there was after irradiation. Hadorn, Rosin and Bertani (1949) suggested that phenol may be specific for certain phenol-sensitive loci in Drosophila. Apart from these claims the great majority of reports of mutagenic action stress the random nature of the mutations induced by the great majority of mutagens. It is interesting to consider the action of sulphur-35 from this aspect. The transmutation of the sulphur-35 atom into an atom of chlorine-35 within the gene strongly suggests that those genes containing sulphur in an essential role would be specifically affected. On the other hand, in the

disintegration of the sulphur-35 atom a weak beta-particle is emitted which Hungate and Mannell (1952) say "is known to be a non-specific mutagen".

Barron and Dickman (1949) have shown that the activities of certain sulfhydryl enzymes are lost by the destruction of the -SH group. Clark et al. (1950) tried to attack the -SH group with specific chemicals, but although mutations were produced, the yield was low. Penicillin, containing as it does a sulphur atom in the molecule, may have some of the steps in its synthesis carried out by sulphur-containing enzymes, some of which may be controlled by genes containing sulphur. It is reasonable to suppose that such genes would be more liable to mutation by sulphur-35 than those not containing this element.

A study involving the mutation of P. patulum by sulphur-35 might have provided a basis of comparison on the possible specific action of the mutagen; but griseofulvin, although it does not contain sulphur, does contain an atom of chlorine in the molecule. The fact that the sulphur-35 atom becomes chlorine-35 on disintegration may invalidate the comparison. The present state of knowledge of the processes causing mutations is not sufficiently advanced to indicate whether the nature of the atom after disintegration could have a bearing on the possible specific action of the mutagen. In both the mutation programmes described, an unexpectedly high mutation rate with respect to antibiotic titre was obtained. Before it can be established

whether this is due to any measure of specificity on the part of the mutagen, similar programmes will have to be carried out using sulphur-35 and involving the mutation of organisms which produce antibiotics not containing either sulphur or chlorine.

Delayed mutation following sulphur-35 treatment.

It is inevitable that when a radioactive isotope is used as a mutagen and more than one atom of the element is assimilated into the gene complex, mutations will occur at various times as the atoms disintegrate. This fact has been observed in the first mutation programme in this thesis where both the mutants which were isolated were found to sector vigorously. This apparent disadvantage can be overcome by subculturing and testing any mutants or variants which may show themselves in the form of sectors. The two mutants of P. patulum, up to the time of the conclusion of this work, had not shown any tendency to produce sectors, although other mutants isolated with them did show this tendency. This may be due to the fact that the original conidium carrying the mutated gene contained only the one atom of the mutagen, or to the fact that any delayed mutations were lost owing to their being of a lethal nature. Delayed mutations are not normal with chemical mutagens or following irradiation because the source is removed on the conclusion of the treatment.

Double mutations.

Closely allied to the previous question is that concerning double mutations, a phenomenon as likely to

occur with radioactive mutagens as with other forms. It must frequently happen in irradiation treatments that two or more mutations are induced within the one nucleus and the same must occur in chemical treatments and also with radioactive isotopes. Up to the present, there is no technique capable of distinguishing with certainty between all types of double and single mutations. If such a technique could be developed, it should be possible, using radioactive mutagens, to find out what proportion of the gene complement of an organism contained atoms of the particular mutagen used. This should be possible because of the fixed rate of decay of a radioactive isotope, and from this, the possibility of telling exactly what proportion of the atoms had changed after a given time. In a mutation programme such as has been described in this thesis, the double or multiple mutant is to be avoided for reasons already given. ~~To achieve this the length~~

INSERT

To achieve this the spores must be removed and cultured individually on normal (S^{32}) medium, after a period of treatment during which the majority of spores have suffered only one mutagenic disintegration (S^{35} -Cl 35). Subsequent mutagenic disintegrations will occur with time but the viable ones will be visible in the form of sectors and can be easily separated.

Medium specific mutants. — related to the phenomenon of aggregation of mutants in certain titre groups, is the fact of medium specificity of certain mutants. It

has been emphasised during the whole of this thesis that mutants displaying an increase or decrease in potential titre do not necessarily repeat this characteristic on a different type of medium. There may be many explanations for this, one of the simplest being limiting factors. It has long been realised that the biosynthesis of a complex molecule, such as an antibiotic, does not take place in one step from simple constituents. Such a molecule is built up in a series of steps involving many intermediate compounds, which may themselves be stages in the synthesis of other end products. In any medium, a limited quantity of raw materials is placed at the disposal of the organism growing upon it. Certain media, for example minimal medium, are rich in simple inorganic materials, whereas others, for example media containing corn steep liquor, are rich in a vast number of more complex substances such as amino-acids, carbohydrates, vitamins etc. (Liggett and Koffler 1948). Further limitations are placed on the synthetic powers of the organism by the efficiency of its own enzyme system together with external factors such as temperature and availability of oxygen. If an organism is mutated in such a way that the synthesis of a particular intermediate compound is speeded up, thereby increasing the yield of the end product, provided the medium and the environment can supply the increased demand for raw materials, that mutant will produce a higher yield of the end product. If, however, this mutant is grown on a medium poor in

this raw material, the increased efficiency of synthesis will be cancelled out by the lack of material and the mutant will produce a lower yield than the parent.

This is a very simple illustration to explain a problem which is in many cases considerably more complicated, involving numerous steps in synthesis. It serves, however, to illustrate the need for medium continuity in this type of mutation programme.

The use of other radioactive isotopes as mutagenic agents. Having shown that sulphur-35 is both an efficient mutagen and an interesting tool with which new techniques might be developed, it is worth considering what other radioactive isotopes might be suitable for use as mutagens. Such isotopes should fulfil certain qualifications if they are to prove satisfactory. Firstly the gene complex of the organism should contain at least one gene in which an atom of the mutagen is represented if mutation is to be caused by the transmutation of the mutagen rather than by any particle emitted during this process. The number of elements present in genes other than carbon, hydrogen, oxygen, nitrogen and sulphur is probably small. Secondly, the isotope should have a conveniently short half-life, for example between about five and two hundred days, a longer half-life would make the treatment inconveniently long. In addition to these, considerable advantage would be gained if the isotope were inexpensive and the radiation not dangerous to the worker, although naturally, precautions can be taken if necessary.

Phosphorus is an element which is found in nuclear material and is common in nucleic acid in the form of phosphate groups. Reuben and Steinglass (1949) used phosphorus-32 as a mutagen on Escherichia coli with practically no result, which suggests that this element is present in the chromosome structure rather than in the genes themselves. This is the only other radioactive isotope that has been tried in this way, namely through being assimilated by the organism itself.

It appears, however, that other radioactive isotopes would be worthy of investigation in this way. Manganese possesses two active isotopes which might be tried. Manganese-52 with a half-life of 6.5 days decays to ~~an excited form of~~ chromium-52 and manganese-54 with a half-life of 310 days decays to an excited form of chromium-54 which itself decays further. Iron has two isotopes, one of which is suitable for trial as a mutagen, iron-59 and iron-55, the former has a half-life of 47 days and the second of 4 years. A mixture of these two isotopes is obtainable from Atomic Energy Research Establishment, Harwell (A.E.R.E. Catalogue of Radioactive Materials and Stable Isotopes.) Zinc and Calcium are two other elements which have suitable isotopes for investigation as mutagens. Zinc-65 has a half-life of 250 days and calcium-45 (180 days) and calcium-41 (8.1 days). Some of the elements mentioned above are in the nature of trace elements, required by the growing organism in very small quantities only. Mutation experiments using these elements in radioactive

form might yield important information on their function in the organism. It is likely that if they are present in the gene complex, relatively fewer genes will contain them, than, for example, an element like sulphur which seems to be more widely distributed.

The high mutation rate observed in the second ultra-violet irradiation of *P. patulum*. As can be seen from the second irradiation of *P. patulum* -Mutant No.2/12 given in histogram E, the mutation rate for titre was considerably greater than in the first irradiation (Strain 1) given in histogram B. Although it is impossible to state exactly the cause of this increase, without carrying out further research, two possible explanations come to mind which may account for it. The simpler one is that in some way the second irradiation may have been more efficient, although care was taken to carry out both these treatments under identical conditions. The second possible explanation is that the first mutant (No.2/12) received a mutation which increased the mutability of other genes. Such mutations have been reported in maize (Rhoades 1941) and in *Drosophila* (Demerec 1937) but apparently not yet in fungi.

The possibility of developing an improved biological assay for griseofulvin. Brian et al. (1951) stated that they believed griseofulvin to be destroyed in non-sterile soil. This strongly suggests the presence of an organism or organisms capable of breaking down the antibiotic in a soil environment ~~because~~^{since} it is

normally a stable substance. It would appear to be worth while investigating this possibility, for if such an organism could be isolated it might be possible by mutation or other means to develop a strain dependent on the presence of the antibiotic for growth. This would enable a biological assay (of the zone of exhibition of growth, type) to be developed.

It may be stated in conclusion, that it has been definitely established that sulphur-35 is a mutagenic agent and that it is satisfactory for mutating certain micro-organisms to increase their antibiotic yield. It is not yet established whether this mutagen is capable of inducing mutants which cannot be produced by other treatments. It does seem likely, that radioactive isotopes, which have received relatively little attention as mutagens considering that they have been available for biological research for nearly a decade, will prove increasingly useful in the future.

SUMMARY.

This work was carried out in an attempt to increase the efficiency of mutation programmes designed to produce strains of micro-organisms giving greater antibiotic yield. The problem was approached from two angles. First, radioactive sulphur (S^{35}), recently shown in America to be a very effective general mutagenic agent for Neurospora crassa, was used to treat two antibiotic-producing fungi, Penicillium chrysogenum (penicillin) and P. patulum (griseofulvin), and with both these species it proved to be an efficient agent for inducing mutations capable of increased antibiotic production. Secondly, methods of screening putative mutants were developed that enable larger numbers of isolates to be screened per unit time than has hitherto been possible by the conventional methods.

Treatment of P. chrysogenum var. brevisterrigma with sulphur-35 consistently yielded isolates 60% of which had mutated in their potential penicillin production and slightly more than 1% of these gave higher yields than the parent strain. A primary screening technique, designed to pick out these higher yielding strains, was evolved. This was based on the principle of using the antibiotic production of the isolate growing on the surface of an agar culture as a measure of its potential yield in submerged fermentation. It is similar to one developed recently in Japan, but avoids the inaccuracies that the Japanese author ascribes to his method by maintaining continuity of the fermentation

medium throughout. By using sulphur-35 as the mutagenic agent, and this surface-culture screening technique, an improved penicillin-producing strain (Strain B) was found among the first 500 isolates tested; on accurate testing it showed a 25-35% improvement on the parent, although it required a slightly richer medium to achieve this increase. A second similar mutation programme with Strain B as the parent produced, among 600 isolates tested, a second mutant (Strain C) capable of the same penicillin yield as Strain B, but not requiring an enriched medium for the purpose. Both these mutants were considered to be potentially useful for industrial production.

With a culture of Penicillium patulum, which produces the antibiotic griseofulvin, there were carried out mutation programmes involving the use of sulphur-35 and ultra-violet irradiation as separate mutagenic agents. As the surface culture screening technique did not lend itself readily to the assay of griseofulvin, one based on a small-volume fermentation was developed. With this technique it was found that sulphur-35 produced a high mutation rate (about 40%) for antibiotic yield of the treated isolates, several of which showed considerably improved yields over the parent. Two of the first 400 sulphur-treated isolates gave, on accurate testing, almost 100% improvement in the yield of griseofulvin. The ultra-violet treatment, ~~the~~ although producing some improved mutants, did not give as many as the sulphur method, nor were those produced as satisfactory as the ones

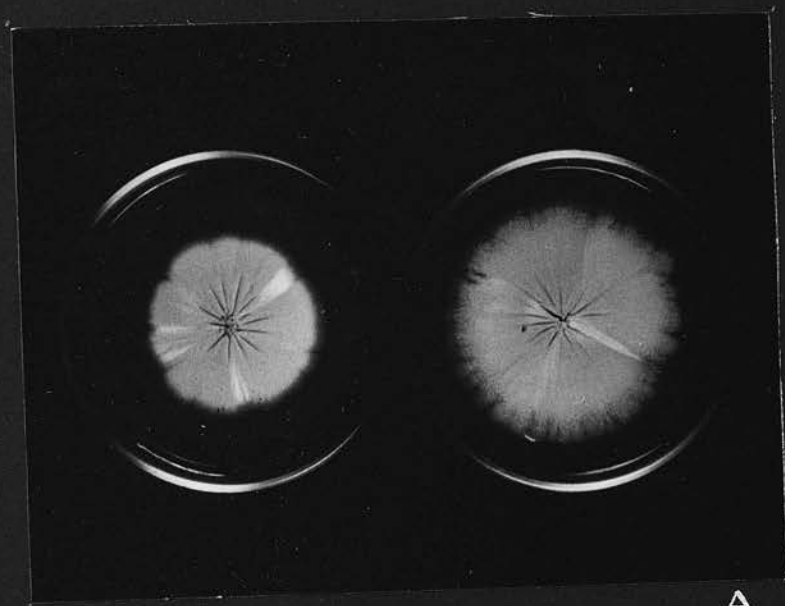
induced by the radioactive isotope.

These results strongly indicate that sulphur-35 is an efficient mutagen for producing strains of fungi yielding increased quantities of antibiotics. This is considered at length in the general discussion. The two primary screening techniques developed have been shown to be at once sufficiently accurate and capable of dealing with greater numbers of isolates than the more conventional methods.

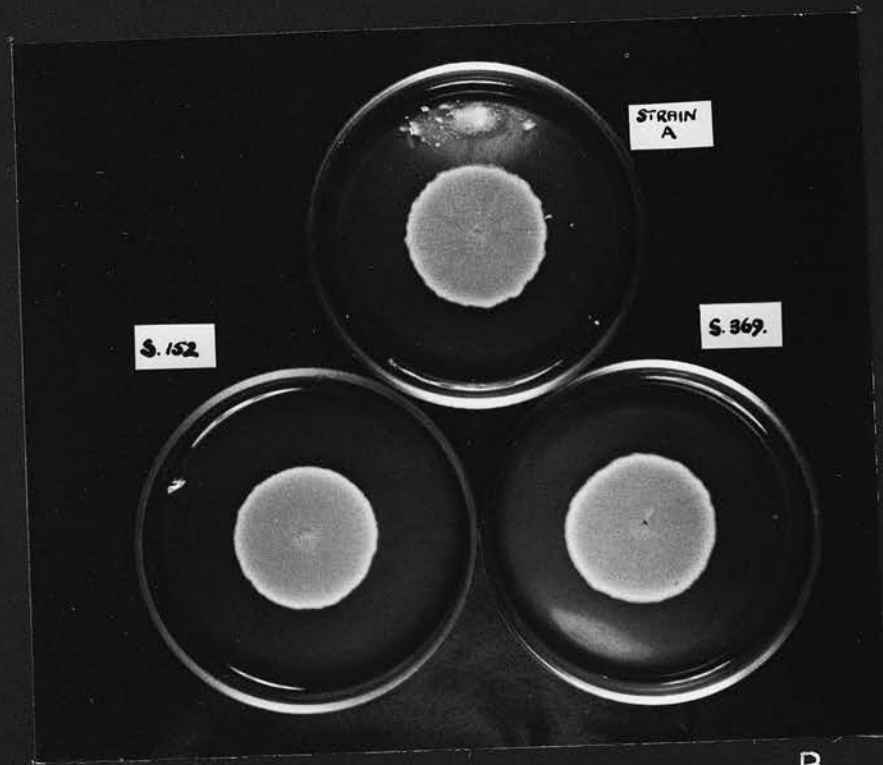
In addition, some preliminary research is described, undertaken to find out the effects of griseofulvin on plant pathogenic fungi of the genus Botrytis. Experiments in vitro and in vivo showed that the presence of the antibiotic upsets the mechanism of hyphal wall-formation in the pathogen, leading in vivo to inability of the hyphae to penetrate the cell membranes of the host.

PLATE I.

- (1) Two giant colonies of P. chrysogenum - Strain **8**. 16 days old (left) and 27 days old (right), showing the tendency to sector vigorously after treatment with sulphur-35. ($2\frac{1}{2}\%$ malt agar).
- (2) Giant colonies of P. patulum - Strain A. and the two high yielding griseofulvin mutants S.152 and S.369 after 13 days growth. This photograph shows the morphological similarity of these three strains. In the case of the two mutants, up to the time of the conclusion of this work, no tendency to form sectors was observed. ($2\frac{1}{2}\%$ malt agar).



A.



B.

APPENDIX A.MEDIA. Details of ingredients and preparation.1. Lactobacillus lactis medium.

Tomato juice filtrate	50 ml
Dextrose	50 g.
Evan's peptone	10 g.
Tween 80	6 g.
Vitamin B ₁₂	10-20 μ g.
Distilled water to	1000 ml.
pH 6.0 \pm 0.1 using NaOH	Agar 15 g.
Autoclave 15 lbs for 7 mins.	

2. Mannitol agar.

Mannitol	10 g.
Lab. lemco.	5 g.
Evan's peptone.	2.5 g.
NaCl.	2.5 g.
Distilled water to	1000 ml.
pH 7.2 \pm 0.1	Agar 20 g.
Autoclave 15 lbs. for 15 mins.	

3. Glucose nutrient agar.

Dextrose	20 g.
Beef extract	10 g.
Evan's peptone	5 g.
NaCl.	5 g.
Distilled water to	1000 ml.
pH 7.2 \pm 0.1	Agar 15 g.
Autoclave 15-20 mins at 15 lbs.	

4. Yeast extract agar.

Difco yeast extract	10 g.
Evan's peptone	10 g.
Distilled water to	1000 ml.
pH 7.3 \pm 0.1	Agar 15 g
Autoclave 15 lbs for 15 mins.	

5. Glycerol agar.

Glycerol	50 ml.
NaCl.	5 g.
Evan's peptone	10 g.
Lab. lemco.	10 g.
Distilled water to	1000 ml.
pH 7.2 \pm 0.1	Agar 25 g.
Autoclave 15 lbs. for 20 mins.	

APPENDIX A. (continued).

6. E. coli maintenance agar.

Benger's casein digest	2.5 g.
Glycerol	2 g.
K ₂ HPO ₄	0.2 g.
Magnesium sulphate (7H ₂ O)	0.2 g.
Ferrous sulphate (7H ₂ O)	1 crystal
Asparagine solution 1.5%	10 ml.
Vitamin B ₁₂ .	25 μ g.
Distilled water to	1000 ml.
pH 7.0 \pm 0.1	Agar 20 g.
Autoclave 15 lbs. for 20 mins.	

7. B. subtilis maintainance agar.

Oxoid peptone	6 g.
Enzymatic casein digest (Benger's)	3 g.
Marmite	3 g.
Difco beef extract	1.5 g.
Distilled water to	1000 ml.
pH 6.6 \pm 0.1	Agar 15 g.
Autoclave 15 lbs for 20 mins.	

8. Nutrient agar.

Lab lemco.	10 g.
Oxoid peptone	10 g.
Na Cl.	5 g.
Distilled water to	1000 ml.
pH 7.2 \pm 0.1	Agar 10 g.
Autoclave 15 lbs for 20 mins.	

9. Bennett's agar.

Bengers casein	2 g.
Difco yeast extract	1 g.
Difco beef extract	1 g.
Distilled water to	1000 ml.
pH 7.5 \pm 0.1	Agar 10 g.
Autoclave 15 lbs for 15-20 mins.	

10. Meat-potato extract agar.

400 g. of peeled potatoes, boil in 1 litre of water for 40 mins. filter through muslin and make up to original volume.

Tryptone	5 g.
Soluble starch	10 g.
Difco beef extract	3 g.
Dextrose	2 g.
K ₂ HPO ₄	1.2 g.
KH ₂ PO ₄	0.8 g.
Ferrous sulphate (7H ₂ O)	1 crystal.
Potato extract to	1000 ml.
pH 6.8-7.0	Agar 15 g.
Autoclave 15 lbs. for 20 mins.	

APPENDIX A. (continued).11. Malt agar.

Malt extract	30 g.
Tap water to	1000 ml.
pH 5.9 \pm 0.1	Agar 25 g.
Autoclave 15 lbs for 20 mins.	

12. Minimal medium agar. (Bonner D. 1946)

Sodium nitrate	3 g.
Potassium di-hydrogen phosphate	1 g.
Magnesium sulphate (hydrated)	0.5 g.
Potassium chloride	0.5 g.
Ferrous sulphate (hydrated)	0.1 g.
Sucrose	30 g.
Distilled water to	1000 ml.
pH natural	Well washed agar 20 g.
Autoclave 15 lbs for 1 min after 7 mins steaming.	

13. Distillers solubles-soya agar-I.

Soya flour	50 g.
Dextrose	25 g.
Distillers solubles	5 g.
Sodium chloride	2.5 g.
Distilled water to	1000 ml.
pH 7.2 \pm 0.1	Agar 25 g.
Autoclave 15 lbs for 20 mins.	

14. Distillers solubles-soya agar-II.

Soya flour	35 g.
Dextrose	10 g.
Distillers solubles	10 g.
Sodium chloride	2.5 g.
Distilled water to	1000 ml.
pH 7.2 \pm 0.1	Agar 25 g.
Autoclave 15 lbs for 20 mins.	

15. C.S.L. agar-(acid).

This medium is subject to very great variation in the quantities of the ingredients and many formulae have been adopted. Each strain of P. chrysogenum, for example, has a different optimum medium. The medium given below is an 'average' medium and may be varied between the values given.

Lactose	25-35 g.
Chalk	10 g.
Corn steep liquor	25-35 g. (total solids)
KH ₂ PO ₄	4 g.
Vegetable oil	2.5 ml.
Distilled water to	1000 ml.

To this must be added one of the known penicillin precursors, such as phenylacetic acid or phenylace-

APPENDIX A. (continued).

tamide etc.

Natural pH 4.2-4.8

Agar 30 g.

Autoclave 15 lbs for 1 min after 7 mins steaming.

In the fermentation medium the agar medium is omitted.

16. C.S.L. agar-(neutral).

As previous, but pH adjusted to 7.2 ± 0.1 .

17. 'Sulphur-free' Minimal agar.

Sodium nitrate 3 g.

Potassium di-hydrogen phosphate 1 g.

Magnesium chloride (hydrated) 0.5 g.

Ferric chloride (anhydrous) 0.1 g.

Sucrose 30 g.

Distilled water to 1000 ml.

pH 6.8-7.2 Well washed agar 20 g.

Autoclave 15 lbs for 1 min after 7 mins steaming.

18. Weindling's medium. (Brian, Curtis and Hemming 1946).

Dextrose 25 g.

Ammonium tartrate 2.0 g.

KH₂PO₄ 1 g.

Magnesium sulphate (hydrated) 1 g.

Minor element concentrate 1 ml.

Distilled water to 1000 ml.

pH to 3.5 Autoclave 15 lbs for 15 mins.

19. Czapek-Dox-peptone medium.

Dextrose 50 g.

Sodium nitrate 2 g.

KH₂PO₄ 1 g.

Potassium chloride 1 g.

Magnesium sulphate - hydrated 0.5 g.

Ferrous sulphate - hydrated 0.01 g.

Distilled water to 1000 ml.

Natural pH Autoclave 15 lbs for 15 mins.

20. Nitro-chalk medium.

Nitro-chalk 4 g.

Brown sugar 60 g.

Muriate of potash 2 g.

KH₂PO₄ 1.5 g.

Magnesium sulphate - hydrated 0.5 g.

pH 6.1-6.2 Autoclave 15 lbs for 15 mins.

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THE EFFECTS OF GRISEOFULVIN

ON CERTAIN PHYTOPATHOGENIC FUNGI.

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THE EFFECTS OF GRISEOFULVIN
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Abstract.

In vitro and in vivo investigations have been carried out into the reaction of actively growing hyphae of Botrytis spp. on encountering an environment containing griseofulvin.

In vitro, the reactions noted were slowing of the growth rate, stimulation to branching, abnormal appearance of hyphal protoplasm and loss of rigidity of the hyphal membrane in the region of the tip. The antibiotic is not translocated within the mycelium.

In vivo, Botrytis tulipae was incapable of penetrating cell membranes in the stem of tulips watered with griseofulvin. The antibiotic was shown to delay or to prohibit the penetration of onion epithelium by B.allii, depending on concentration. The characteristic curling and stunting of hyphae caused by griseofulvin have both been observed to occur in plant cells.

INTRODUCTION

Griseofulvin, first isolated by Oxford, Raistrick & Simonart (1939), and 'curling factor', by Brian, Curtis & Hemming (1946), have been shown to be identical (Grove & McGowan, 1947) (Brian, Curtis & Hemming, 1949). By reason of its marked fungistatic properties, griseofulvin has been the subject of several investigations that have shown it to possess a number of properties

desirable in a substance intended to be effective in controlling plant diseases. Brian (1949) has shown that the antibiotic is stable over a wide pH range, is not affected by autoclaving and is fungistatically active against all groups of fungi except the Oomycetes, yeasts and Actinomycetes. Brian, Wright, Stubbs & Way (1951) demonstrated that it was systemic in plants, being taken in by the roots of lettuce and oats and translocated to the leaves and remaining detectable in the plant for some time after its source was removed. These authors also achieved control of Botrytis cinerea on lettuce and Alternaria solani on tomatoes, but found that griseofulvin is quickly degraded in unsterile soil. This communication gives a preliminary report of a research project designed to study the mode of action of griseofulvin, in relation to the control of plant disease.

PART I.

'IN VITRO' EFFECTS.

Because of Brian's (1949) detailed description of the effects of griseofulvin on the germination and the resultant hyphae of Botrytis allii when grown on a medium containing this substance, an investigation has been carried out into the reaction of hyphae, previously grown in a normal environment, on suddenly entering one containing griseofulvin. Such a situation could occur in the event of a fungal spore germinating on a leaf epidermis and meeting on penetration an effective con-

centration of the antibiotic within the plant cells.

Method.

The method used (parallel hanging drop cultures) follows closely that previously described (Aytoun, 1953), except that a Winge micromanipulation cell (Winge 1935) was used instead of the normal glass rings of a van Tieghem cell, in order to obtain better aeration. For preparing the hanging drops the medium used throughout was 2½% malt agar, filtered and with pH adjusted to 5.5. The griseofulvin was dissolved in acetone and added to the liquid malt agar at between 45°-55°C., so as to give an acetone concentration of not more than 4% in the medium.

The Winge cell, its arms plugged with cotton wool, was fixed to a microscope slide by means of a beeswax and soft paraffin mixture, and 2 ml. of sterile water was added to provide moisture. Two malt agar drops, one containing the required concentration of griseofulvin, the other free from it, were placed on a No.1 coverslip so that their edges were close together but not touching. The drop not containing the antibiotic was inoculated with a few spores of the fungus and the coverslip was inverted and sealed to the Winge cell with soft paraffin. Control cultures were also set up with one of the hanging drops containing acetone at the same strength as was present in the griseofulvin drops. Each culture was placed in a sterile Petri dish and incubated.

By this method the fungus became established on the inoculated agar drop and eventually grew across and impinged on the agar containing griseofulvin, where its reactions were observed.

Observations on Botrytis spp.

This work has been carried out using Botrytis spp. because the genus is responsible for disease in a great variety of plants. Broadly speaking, by this method the reactions of the Botrytis spp. tested (B.cinerea, B.allii, B.tulipae and B.fabiae) to griseofulvin are identical and their response to concentration is similar to that described for B.allii by Brian (1949).

There are four ways in which the reaction of the fungal hypha to a griseofulvin-containing environment shows itself.

(1) Decrease in growth rate.

At concentrations between 0.01 and 0.1 $\mu\text{g/ml}$. reduction in growth rate can be detected without curling of the hyphae. Concentrations between 0.1 μg . and 1.0 g/ml . cause the hyphae to curl in the characteristic manner, and at about the latter concentration stunting of the hyphae can be seen. This stunting increases with greater concentrations of griseofulvin, until at about 10 $\mu\text{g/ml}$. other severe growth abnormalities appear. These take the form of giant cells, PLATE 1D, (mentioned by Brian, 1949), globular excrescences that do not develop further, a thalloid type of growth resembling in form the fronds of Fucus

and bursting of the hyphae, PLATE 1E. There is a difference in reaction to concentration, shown by hyphae growing on the surface of the agar, aerial hyphae and those growing through the agar. Those growing embedded in the medium show a more severe reaction than those on the surface. Aerial hyphae arising from mycelium growing on distinctly high concentrations of the antibiotic appear normal. If the fungus is first established on a griseofulvin-free medium, it is able to continue growing in concentrations of griseofulvin in which spores would merely germinate and stop growing. This has been observed when a few spores have been accidentally scattered on to the griseofulvin hanging drop during inoculation. The spores germinating in the presence of 10-20 $\mu\text{g/ml}$. of the antibiotic produced a short germ tube only, whereas hyphae growing across from the other drop have been seen to grow slowly through them.

(2) Stimulation to branch coupled with loss of direction.

A normal fungal hypha on encountering a new nutritional environment forms branches that direct themselves forwards and outwards in an attempt to grow over the environment, PLATE 1A. In this sense a fungus may be said to show a directional trend in its growth. On entering an environment containing more than $\approx 1\mu\text{g/ml}$. of griseofulvin, it forms more than the normal number of branches, and these do not show any directional trend; frequently growing round towards their point of origin,

they may aggregate to form a tight knot, PLATE 1B & C. At concentrations between 0.1-1.0 μ g/ml. hyphae, though showing the typical curling form, in general maintain the normal direction of growth.

(3) A typical appearance of hyphal protoplasm.

Healthy fungal hyphae, when viewed through a microscope by transmitted light, appeared in the region of the growing tip to be almost homogeneous in content. The tip region of hyphae showing stunting appeared more granular. Small dark bodies of the order of 1 μ in size were frequently seen, singly or in groups of up to 6 or 8. They were seen to be in motion if watched for periods of 30 seconds or more. This movement was random, either as a complete group, or individually, in any direction. Attempts have been made to correlate the presence and position of these granules with the formation of hyphal abnormalities, but so far unsuccessfully. These granules are not obvious in curling hyphae.

(4) Disturbances in hyphal wall rigidity.

Hyphae growing in contact with griseofulvin tend to be larger and more uneven in diameter than in controls, whose diameters are remarkably constant. The abnormalities that appear with the higher concentrations of griseofulvin may be swellings or bulbous excrescences on one side or involving the whole of the hypha, PLATE 1D. They are formed initially close to the growing tip and have not so far been observed

forming further back than the first septum from the tip. Burst hyphae are frequently seen, PLATE 1E, the bursts occurring in the region of the growing tip or, rarely, at the junction of a branch. The quantity of protoplasm expelled on bursting suggests that an appreciable pressure existed within the hypha before rupture.

DISCUSSION.

As a result of these in vitro observations, it is possible to draw certain conclusions as to the way griseofulvin affects the hyphae of species examined. In the first place the fungus in the form of the young germ tube is the most severely affected by the antibiotic. The actively growing hypha is able to maintain a restricted degree of growth in concentrations of the antibiotic that would prohibit spores from developing beyond the germ-tube stage. Secondly, to judge from the normal form of aerial hyphae produced from mycelium in contact with and severely affected by relatively high concentrations of the antibiotic, it is clear that griseofulvin is not translocated within the mycelium (suggested by Brian, 1949). It is possible to go further than this and say that the effect of a given concentration of griseofulvin on a hypha is proportional to the area of the hypha at the growing point in contact with it. Individual hyphae change their degree of reaction to the antibiotic on growing into the agar medium after growing on the surface, and vice versa. Finally, the antibiotic appears to exert its

effect on the hypha in the region of the tip. Initiation of swellings and other deformities almost always takes place at the point just behind the tip.

This effect on the hypha in the region of the growing point may be caused in either of two ways. Either the antibiotic influences the formation of the hyphal wall in such a way that extension is inhibited, thereby causing a build up of pressure inside the hypha, which results in swelling or bursting, or the presence of the antibiotic causes abnormally weak areas in the wall, which are unable to withstand the pressures of normal turgor. At the present stage of the work it is impossible to decide between these two explanations, but it is hoped to study this problem in the near future.

PART II.

INVESTIGATIONS IN VIVO

INTRODUCTION.

An attempt was made in these experiments to obtain control of tulip fire (Botrytis tulipae) by building up a concentration of griseofulvin within the plant and then to cut sections and examine the tissues and hyphae microscopically to find out how the fungus was being controlled. Other experiments were carried out with onion epithelium to test the power of penetration of Botrytis allii in the presence of various concentrations of the antibiotic.

METHOD.

METHOD.

Six Darwin tulips of the same (unknown) variety, which had been grown outside to the stage of a two inch (5 cm.) green spike, were transferred with a small ball of soil into 6 inch pots containing a vermiculite-sand mixture (equal volumes) and placed in a warm greenhouse. The bulbs were watered three times weekly, once with tap water and twice with a liquid feed prepared from Mullard Nutrient Pdr. M.15 as supplied by Mullard Horticultural Eng. Co. Ltd., Egham, Surrey. The bulbs were grown on to the green bud stage (approx. 5 weeks), during which period three of the bulbs were each watered 7 times with 50 ml. of water containing 85 μ g/ml. of griseofulvin, previously dissolved in a small quantity of acetone. The addition of the griseofulvin in acetone to the water was made immediately before pouring the solution onto the soil surface. The leaves were not wetted by the solution. The control plants were each watered with 50 ml. of tap water containing the same concentration of acetone as the griseofulvin watered plants (0.85%). Watering with the griseofulvin and control solution was carried out on days when normal watering and feeding were not being done.

At the green bud stage all the plants were severed, by means of sterile razor-blade, from the bulb at a point about 50-60 mm. above its tip. A drop of water was added to the cut surface of all the plants remaining

in the pots and these surfaces were inoculated with spores of B. tulipae obtained from an infected plant. The plants were then transferred to a "Perspex" humidity chamber in the greenhouse for 48 hours.

The upper parts of the plants were used to assay the griseofulvin in them. This was done by cutting off portions of the stem of each plant and, after weighing them, grinding them up with ten times their own weight of distilled water. The liquid so obtained was filtered and assayed with B. allii, as described by Brian, Curtis & Hemming (1946). This method was used in preference to the improved method described by Crowdy & Premer (1955), because it was thought that grinding up in water might give a truer measure of the free griseofulvin in the plant tissues. There is no evidence that bound griseofulvin would have the same effect on the invading fungus. Assayed in this way the upper stem of the treated tulips showed a concentration of between 2 and 3 $\mu\text{g/g}$. (live wt.). The control plants on assay showed no trace of griseofulvin.

After 48 hours in the damp chamber the plants were removed: judging by the discolouration of the stems of the control plants, the fungus had advanced 28 - 34 mm. down the stems. The treated plants showed a shallow brown ^{ring} ~~ring~~ extending 2 - 3 mm. down the side of the stem, and this proved to be the total advance made by the pathogen.

The pieces of stem were fixed in Karpechenko's

solution and embedded in paraffin wax; longitudinal sections were then made and stained in cotton-blue lactophenol. Sections of the control plants showed that the spores had germinated and the hyphae had grown down into the stem, passing freely from cell to cell, PLATE 2F. There was some evidence of hyphae growing down xylem vessels, but this seemed to be unusual. Some distance behind the advancing hyphae the mycelium consolidated into a compact mass, which moved down the stem, leaving behind it complete cellular disintegration.

Sections from the treated plants showed that after germination the hyphae had grown down into the stem, gaining access to the cut cells at the surface. The griseofulvin in these cells was probably diluted by the drop of water placed on the cut surface. In the main the hyphae of B. tulipae were halted at the line of the first or second end walls of cells they encountered, and few hyphae were found beyond this region, PLATE 2J. Some degree of penetration had taken place from the pathogen's growing through the intercellular spaces, and this probably accounted for the pieces of mycelium found below the general line at which the fungus was halted. The xylem vessels were not used on this occasion, presumably because the concentration of griseofulvin was higher in them than in the surrounding tissues.

Individual hyphae of B. tulipae on encountering the walls of cells were usually turned by them, where-

upon the hyphae proceeded to grow and form a spiral round the inside of the cell, until it became more or less full, PLATE 2L. In some instances the hyphal tip on meeting the cell membrane stopped growing and a swelling formed a short distance behind the tip, PLATE 2K. This was often followed by branching further back, some of the branches growing round inside the cell and filling it up. Both stunting and curling of hyphae were seen in these sections, suggesting that, although the concentration of griseofulvin within the stem may have varied, the overall effective concentration of the antibiotic must have been in the neighbourhood of the transition concentration between curling and stunting. In vitro for B. tulipae, this is in the region of 1 μ g/ml., which confirms the figure obtained in the assay of different portions of the stem.

PENETRATION EXPERIMENTS USING ONION EPITHELIUM.

Experiments were conducted to test the powers of B. allii to penetrate onion epithelium in the presence of griseofulvin. Onion epithelium was used because it can be stripped off the scales in large sheets, which are uniformly one cell thick. It can be easily handled without tearing or breaking. Further, the inside (adaxial) epithelium can be used, and it has no stomata or other openings that might allow false penetration. It was found that the 'toughness' of the epithelium decreased somewhat on the scales nearer the centre of the bulb and therefore the epithelium from

one scale was used for one series of experiments. To provide the maximum amount of material from one bulb scale, large Spanish type onions were used.

These experiments were carried out in two ways. The first was to strip off several pieces of epithelium, approx. 2 cms. wide and 6-8 cms. long, and lay them in Petri dishes previously prepared with 2 $\frac{1}{2}$ % malt agar containing known concentrations of griseofulvin. The inside surface of the epithelium was placed on the agar and smoothed flat with a sterile wire loop. The surface of the onion tissue was inoculated with B.allii spores and incubated at 24°C. until required. The second method was to cut slices of onion scale 2.5 cms. wide complete with epithelium and to soak the scale in sterile saline containing the desired strength of griseofulvin. After soaking, the pieces of scale were washed in saline for 1 minute and dried with sterile filter paper; the concave surfaces were inoculated with B. allii spores. The pieces were placed in Petri dishes with damp cotton wool and incubated as described above. Controls were prepared by soaking pieces of onion scale in saline containing no griseofulvin.

After incubation the epithelium was stripped off the surface of the agar and the onion scales were stained in cotton-blue lactophenol and examined microscopically. The agar surface and the surface of the scale from which the epithelium had been removed were

also stained. Any mycelium that had penetrated the epithelium and grown either into the agar or into the tissue of the onion scale, was left behind on removal of the epithelium and could be seen under the microscope. This together with examination of the stained epithelium provided an accurate assessment of the penetration, as well as information on the behaviour of the germ-tubes when inside the epithelial cells in the presence of griseofulvin.

The method involving placing the epithelium on the agar worked satisfactorily. The cells were not plasmolysed and ~~the~~ remained healthy for at least three days. The difficulty with the method involving the whole onion scale lay in the length of time the tissue had to be soaked in griseofulvin solution to get an even distribution of the antibiotic within the tissues, from 24-48 hours. The variation appeared to be due to the difference in the condition of the scales: consequently all pieces were soaked for 48 hours. In spite of this, complete uniformity was not always achieved. Generally, however, the results obtained by the two methods were closely similar.

The concentrations of griseofulvin tested in these experiments and the results obtained are shown in Table I.

TABLE I

Penetration of onion epithelium by B. allii
in the presence of griseofulvin.

Griseofulvin $\mu\text{g/ml.}$	12 hours	24 hours	48 hours	72 hours
30	-	-	-	-
15	-	-	-	-
10	-	-	+	+
5	-	-	+	++
3	-	+	++	++
1	+	+	++	++
0.3	+	++	++	++
0.0	++	++	++	++

- = no sign of penetration.

+ = a few isolated cases of penetration.

++ = frequent penetration.

+++ = vigorous penetration.

These results show that under the above conditions a concentration of between 10 and 15 $\mu\text{g/ml.}$ of griseofulvin prevents the hyphae from penetrating the epithelium for three days. Lower concentrations cause delay for varying periods. After germination, facile penetration of the outer cell membrane occurs. Once inside the cell at griseofulvin levels of 3-5 $\mu\text{g/ml.}$ and above, development of the fungus is severely reduced. At about 1.0 $\mu\text{g/ml.}$ the intra-cellular hyphae are somewhat stunted but growth continues at a

reduced rate. Concentrations below this cause curling, but penetration does not appear to be affected.

DISCUSSION.

Results both from in vitro and in vivo experiments have shown that the fungal hypha is profoundly modified when growing in physical contact with griseofulvin. These modified hyphae are less able to penetrate plate cell membranes than those growing in the absence of griseofulvin.

Systemic concentrations of the antibiotic only sufficient to cause curling of Botrytis hyphae may not be capable of giving full protection to the plant against these pathogens. If, however, a concentration of griseofulvin can be built up inside the plant sufficient to cause stunting ($> 1 \mu\text{g/ml.}$), it would seem that protection of the plant against attack from Botrytis would be achieved. The presence of the antibiotic within the cells of the plant allows spore germination and possible penetration of the epidermal layers by a pathogen, but would, if the concentration were sufficient, restrict that pathogen to a few adjacent cells. Griseofulvin applied by spray would act on the fungus on germination, the stage at which it is most vulnerable to the antibiotic. Information on the amount of griseofulvin absorbed by a plant sprayed with it would be valuable.

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SUMMARY.

1. In vitro and in vivo experiments have been carried out to find out how griseofulvin affects the growing hyphae of Botrytis species.
2. In vitro experiments to study the reaction of growing Botrytis hyphae on entering a griseofulvin environment have shown that the antibiotic only acts on a small region of the hypha around the tip and that it is not translocated within the mycelium. Some modification of the protoplasmic content of hyphae takes place together with loss of rigidity of hyphal membranes.
3. In vivo experiments, involving control of the spread of B. tulipae in tulip plants by griseofulvin taken into the plant via the roots, showed on sectioning the tissues that the fungus is unable to pass from cell to cell through the cell membranes in the presence of the antibiotic and so is unable to spread through the plant.
4. Experiments to test the ability of B. allii to penetrate onion epithelium in the presence of

griseofulvin showed that penetration is delayed for 3 days by 10-15 $\mu\text{g}/\text{ml}$. of the antibiotic.

Hyphae within the epithelial cells are profoundly modified in form.

5. All the hyphal modifications observed in vitro, such as curling, stunting and bursting of hyphae, have been observed in vivo.

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EXPLANATION OF PLATES.

Illustrating R.S.C. Aytoun's article on
'The Effects of Griseofulvin on Certain Pytopathogenic
Fungi'.

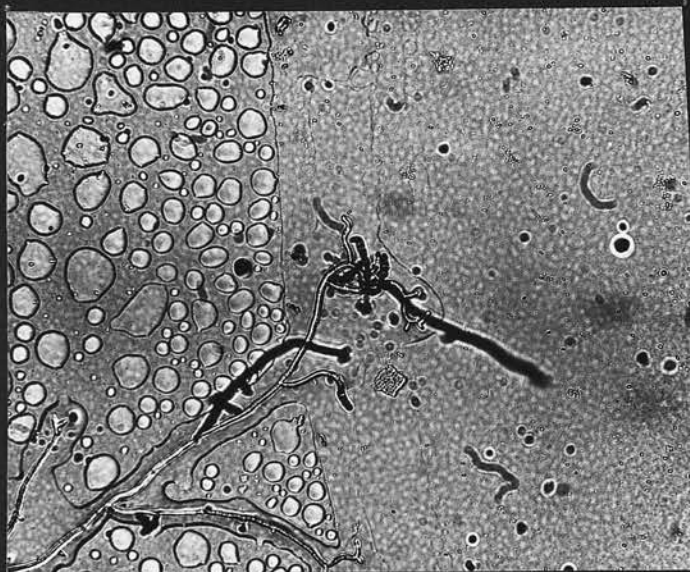
(Note: The magnifications given are approximate.)

Plate I 'in vitro'

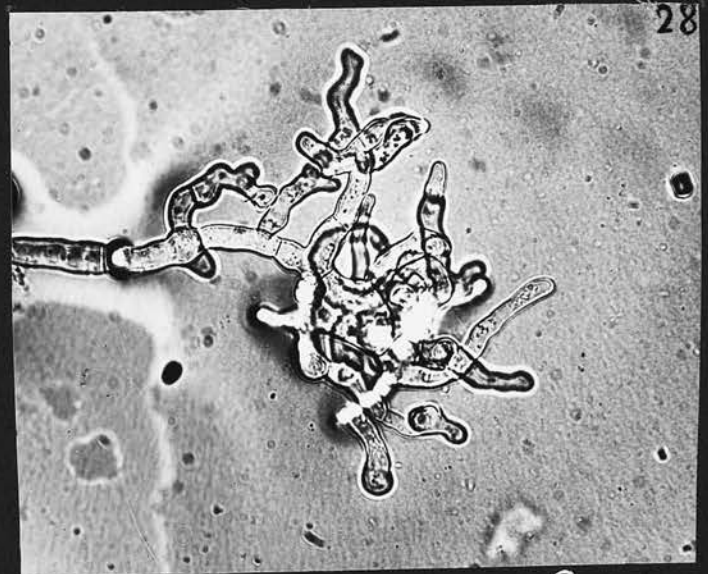
- A. Hypha of Botrytis tulipae entering a malt agar hanging drop containing no griseofulvin. Note rate of branching. (x 100)
- B. Hypha of B. tulipae entering a hanging drop containing 1.6 μ g/ml. of griseofulvin. Showing early stimulation to branch with loss of direction of growth. Dark aerial hyphae not in contact with the medium appear normal. (x 100)
- C. Hypha of B. tulipae entering a hanging drop containing 12 μ g/ml. of griseofulvin. Shows immediate stimulation to profuse branching. Hyphae have granular contents and are of uneven thickness. (x 500)
- D. Hyphae of B. cinerea after growing for 48 hours in an environment containing 11 μ g/ml. of griseofulvin. Shows breakdown in rigidity of hyphal walls causing random swelling of the mycelium, with granular contents. (x 500)
- E. Showing the typical bursting of a hypha near the tip, frequently seen when hyphae are in contact with concentrations of griseofulvin of about 10 μ g/ml. or more. (x 500)



A.



B.



C.



D.



E.

Plate II 'in vivo'.

- F. Hyphae of B. tulipae advancing down inside the stem of an untreated tulip. Hyphae are straight and pass easily from cell to cell.
(Compare with G). (x 500)
- G. Section of tulip stem treated with griseofulvin and containing about 2.0 μ g/gm. live weight. Advancing hyphae of B. tulipae show obvious curling and twisting. (x 500)
- H. Section of untreated tulip stem at the cut surface inoculated with B. tulipae. Hyphae are seen throughout the section and are able to pass readily from cell to cell.
(Compare with J). (x 100)
- J. Section of treated tulip stem at the cut surface where inoculation took place. Advance of B. tulipae is stopped by first end walls of cells, which it cannot penetrate. (x 100)
- K. Showing hyphae of B. tulipae stopped by the end wall of a cell in the stem of a treated tulip. Note swellings just behind the tips of some hyphae. (x 500)
- L. Cell in stem of tulip treated with griseofulvin that has contained the pathogen. Hyphae of B. tulipae have almost filled the interior of the cell and are unable to break out. (x 500)



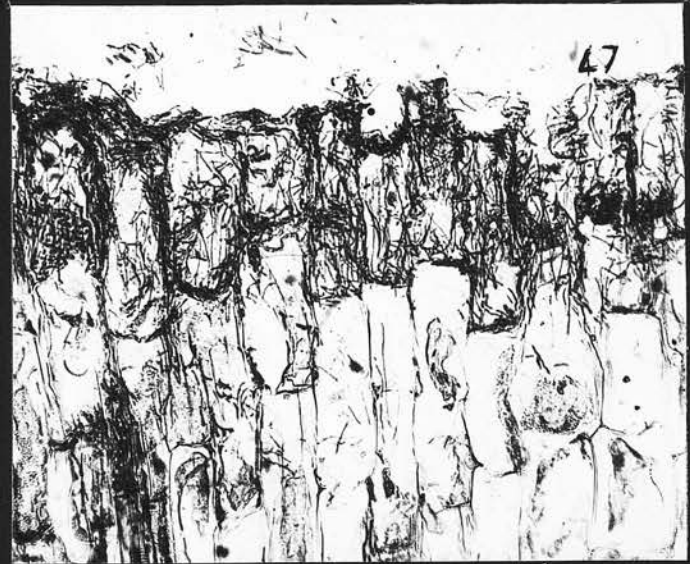
F.



G.



H.



J.



K.



L.